CARDIOVASCULAR EFFECTS OF ASYMMETRIC DIMETHYLARGININE

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Abstract

Nitric Oxide (NO) is an important mediator of cardiovascular function and its impaired synthesis is a feature of many cardiovascular diseases. Raised concentrations of an endogenous inhibitor of NO synthesis, asymmetric dimethylarginine (ADMA), are associated with renal failure, hypertension, heart failure and impaired angiogenesis. ADMA is generated during protein turnover and metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). The significance of this pathway *in vivo* is unknown.

This series of studies demonstrates that the metabolism of ADMA by DDAH influences NO synthesis *in vitro* and *in vivo* and that this pathway is likely to be important in man. An important regulator of tissue development and remodelling, all-*trans*-retinoic acid (atRA), has been shown to upregulate an isoform of DDAH, DDAH2, and regulate NO synthesis *in vitro*. Using a novel transgenic mouse model, it has been shown that the overexpression of DDAH *in vivo* can increase NOS activity and lower blood pressure. A mouse model of unilateral hindlimb ischaemia has been used to demonstrate that ischaemia can increase ADMA formation and also upregulate DDAH expression in order to restore ADMA levels to baseline and potentiate NO synthesis. Finally, a randomised, double-blind, placebo-controlled study demonstrates that an acute systemic increase in ADMA produces adverse cardiovascular effects in humans, both at rest and during exercise. These results support a causal role relationship between raised ADMA levels and cardiovascular dysfunction. The data also indicates that ADMA is metabolised by DDAHs

extensively in humans *in vivo*; humans generate approximately 300μmol of ADMA per day, of which approximately 250μmol is metabolised by DDAHs.

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Table of Contents

Chapter One: Introduction

1.1 Nitric Oxide 7

1.2 Animal Models of Cardiovascular Disease 13

1.3 Arginine Paradox 14

1.4 Asymmetric Methylarginines 15

1.5 Biological and Clinical Significance of ADMA 17

1.6 Methylarginine Formation 20

1.7 Methylarginine Metabolism 22

1.8 Summary 27

Chapter Two: Methods

2.1 Molecular and Cellular Biology 30

2.2 Biochemical Assays 37

2.3 Mouse Studies 41

2.4 Human Study 46

2.5 Statistics 47

Chapter Three: Retinoic acid regulates NO synthesis and ADMA metabolism

3.1 Introduction 49

3.2 Protocols 50

3.3 Results 51

3.4 Discussion 59

Chapter Four: DDAH overexpression in a transgenic mouse model regulates NO synthesis and lowers blood pressure

4.1 Introduction 64

4.2 Protocols 65

4.3 Results 66

4.4 Discussion 73

Chapter Five: ADMA metabolism by DDAH regulates NO synthesis following hindlimb ischaemia

5.1 Introduction 77

5.2 Protocols 78

5.3 Results 78

5.4 Discussion 86

Chapter Six: Cardiovascular effects of ADMA in humans

6.1 Introduction 92

6.2 Protocol 94

6.3 Results 95

6.4 Discussion 101

Chapter Seven: Discussion 108

Chapter Eight: References 116

Supplements 141

Chapter One: Introduction

1.1 Nitric Oxide

1.1.1 Nitric Oxide Synthesis

Nitric oxide (NO) is an important mediator of cardiovascular function in man (Vallance *et al.*, 1989; Moncada and Higgs, 1993). Furchgott and Zawadzki were the first to suggest that an endothelium derived relaxing factor (EDRF) might be responsible for acetylcholine induced endothelium dependent vascular relaxation (Furchgott and Zawadzki, 1980). EDRF was subsequently identified as NO (Ignarro *et al.*, 1987; Palmer *et al.*, 1987). In the same year, Hibbs and colleagues demonstrated the cellular conversion of arginine to citrulline, nitrite and nitrate, and showed that this process could be blocked by an arginine analogue, NG-monomethyl-L-arginine (L-NMMA; Hibbs *et al.*, 1987). This in turn led to the discovery of a family of NO generating enzymes, nitric oxide synthases (NOS). Three genetically distinct NOSs share a similar catalytic mechanism requiring molecular oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), and the essential cofactors, flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), and tetrahydrobiopterin (BH4) (reviewed in Alderton *et al.*, 2001). Endothelial NOS (eNOS; NOS3) and neuronal NOS (nNOS; NOS1) are constitutively expressed enzymes which are calcium dependent and produce small amounts of NO (Palmer *et al.*, 1988; Bredt et al., 1990). A third inducible isoform, inducible NOS (iNOS; NOS2), is calcium independent and produces large quantitites of NO rapidly in response to inflammation and injury (Stuehr *et al.*, 1991). The mechanisms that regulate NO synthesis by these enzymes are complex and play a fundamental role in the regulation of cardiovascular, neurological, and immune functions. Disruption of NO synthesis has been implicated in the pathogenesis of a range of cardiovascular

diseases making this pathway a potential target for cardiovascular therapy (Cooke and Dzau, 1997; Napoli and Ignarro, 2001).

1.1.2 Regulation of NO synthesis

Alterations in the balance between NO formation and degradation can produce rapid changes in NO concentrations. NO is rapidly inactivated by its interaction with reactive oxygen intermediates (Stamler *et al.*, 2001). In some situations such as the control of basal blood flow, NO formation must be maintained at an almost constant level. In other settings, the NO signal must be short lived or pulsatile.

eNOS and nNOS tend to be regulated at the post-transcriptional level. Two important physiological stimuli for continuous NO formation by endothelial cells are fluid shear stress (in the physiological range of 10-40 dynes/cm^2) and pulsatile stretch (Cooke *et al.*, 1991). As well as triggering the immediate release of NO, laminar flow also increases the expression of eNOS (Uematsu *et al.*, 1995) and superoxide dismutase (which reduces the oxidative degradation of NO; Topper *et al.*, 1996).

Dimerisation of NOS generates high affinity binding sites for arginine and the essential cofactor BH4 as well as allowing the transfer of electrons from reductase to oxygenase domains of the enzyme. The presence of calmodulin enhances NOS synthesis by increasing electron flow. Calmodulin is tightly bound to iNOS whereas the binding of calmodulin to eNOS and nNOS is dependent on intracellular calcium levels (reviewed in Alderton *et al.*, 2001). Acetylcholine (ACh) and a number of circulating factors (including bradykinin, thrombin and vascular endothelial growth factor; VEGF) act via cell surface receptors to alter endothelial calcium

concentrations and trigger NO formation. Specific protein kinases including Akt regulate eNOS activity by the phosphorylation of critical residues (Ser1179; McCabe *et al.*, 2000). Scaffolding proteins including caveolin and heat shock proteins (hsp90) interact with eNOS to form a signalling complex at the endothelial cell surface in close proximity to the y+ (CAT1) arginine transporter (McDonald *et al.*, 1997), arginosuccinate synthase and arginosuccinate lyase (Flam *et al.*, 2001). Recent interest has focused on the inhibition of all three NOS isoforms by endogenous, structural analogues of arginine, and in particular asymmetric dimethylarginine (ADMA; Vallance *et al.*, 1992).

1.1.3 NO as a signalling molecule

NO acts as a rapidly formed, rapidly acting, and rapidly inactivated signal within cells as well as between neighbouring cells. NO produced by endothelial cells, for example, crosses to neighbouring vascular smooth muscle cells (VSMC) and activates soluble guanylate cyclase (sGC) by binding to a haem moiety within the enzyme (Ignarro *et al.*, 1999). This triggers the formation of cyclic guanosine monophosphate (cGMP) which in turn accounts for many of the physiological effects of NO via a cGMP-dependent protein kinase (Arnold *et al.*, 1977). sGC is most abundant in smooth muscle cells (including VSMC), neurones, platelets, and cardiomyocytes suggesting that these represent important targets for NO action. NO also regulates proteins by a direct reversible interaction with reduced thiol groups; the S-nitrosation of specific cysteine residues can result in the regulation of ion channels, G proteins, and transcription factors (Stamler *et al.*, 2001).

NO has a very short half life due to its interaction with other free radicals. NO reacts rapidly with superoxide to form peroxynitrite, a powerful oxidant which in turn modifies proteins and lipids by nitration. This highlights the concept that NO signalling is intimately related to oxidative stress and the redox status of the cells involved. There is also evidence from *in vitro* studies to suggest that BH4 and/or arginine depletion can lead to uncoupling of oxygen reduction and arginine oxidation, resulting in superoxide formation by NOS (Pritchard *et al.*, 1995; Wever *et al.*, 1998).

1.1.4 NO regulates the cardiovascular system

NO regulates a number of specific cardiovascular functions including vascular tone, blood pressure, heart rate and cardiac contractility. Pharmacological NOS inhibitors such as L-NMMA have played a key role in the investigation of the physiological role of NO in the regulation of blood flow and blood pressure in animals and man. The endothelium dependent relaxation of vascular rings in organ bath studies can be blocked by L-NMMA (Palmer *et al.*, 1988). In animals, systemic doses of L-NMMA increase blood pressure and produce a reflex bradycardia (Rees *et al.*, 1989). In humans, forearm arterial infusion of L-NMMA reduces forearm blood flow indicating that NO is an important local determinant of resting tone in human arterioles (Vallance *et al.*, 1989). Systemic doses of L-NMMA raise blood pressure, reduce cardiac output and increase systemic vascular resistance in healthy volunteers as well as in patients with sepsis (Petros *et al.*, 1991; Haynes *et al.*, 1993).

In the absence of isoform specific NOS inhibitors, one approach to studying the roles of specific NOS isoforms has been the creation of genetically engineered mice. Aortic rings from mice lacking eNOS display no relaxation in response to acetylcholine, are

hypertensive with mean arterial blood pressures 20 to 30 mmHg greater than wild type values, and develop left ventricular hypertrophy (Huang *et al.*, 1995; Shesely *et al.*, 1996). Conversely, transgenic mice overexpressing bovine eNOS under the control of an endothelium specific promoter are hypotensive (Ohashi *et al.*, 1998). eNOS overexpressing mice also demonstrate reduced vasorelaxation in response to exogenous NO which may be a result of nitrate tolerance following chronic eNOS overexpression, the mechanisms of which are unclear.

In addition to the above, NO regulates cellular growth, proliferation, adhesion and apoptosis (Cooke and Dzau, 1997; Napoli and Ignarro, 2001). These effects may mediate the regulation of cardiac remodelling as well as vascular remodelling during angiogenesis by NO. The association between impaired NO synthesis and atherosclerosis (as well as restenosis following vascular injury) may be explained in part by the loss of these control mechanisms.

1.1.5 Clinical significance of NO

Impaired endothelium-dependent vasorelaxation (synonymous with endothelial dysfunction) is implicated in the pathogenesis of many cardiovascular disorders including hypertension, atherosclerosis and restenosis following angioplasty (Ludmer *et al.*, 1986; Vita *et al.*, 1990; Weidinger *et al.*, 1990). The dysfunctional endothelial cell is unable to support vascular smooth muscle cell relaxation, promotes leukocyte adhesion, and cannot inhibit VSMC proliferation. The loss of NO bioavailability is an essential feature of endothelial dysfunction. Endothelial dysfunction is also characterised by an increase in endothelium derived contracting factors, adhesion molecule expression and increased superoxide formation.

Endothelial vasodilator function can be assessed as the ability of a vessel to dilate in response to an endothelial stimulus. Angiography can demonstrate epicardial coronary artery vasodilation in response to an intracoronary infusion of ACh *in vivo*. ACh triggers the release of NO by binding to endothelial muscarinic receptors in a normal vessel. When endothelial function (and NO synthesis) is impaired, a direct action of ACh on vascular smooth muscle is unmasked and a paradoxical vasoconstriction is observed in conduit arteries and veins (Ludmer *et al.*, 1986). Impaired endothelium-mediated, NO-dependent vasodilation is associated with traditional cardiovascular risk factors even in the absence of atherosclerosis (Vita *et al.*, 1990; Zeiher *et al.*, 1993). The development of noninvasive methods to assess endothelial function has allowed larger studies of healthy subjects and has confirmed independent associations of endothelial dysfunction with age, hypercholesterolemia, cigarette smoking, and diabetes mellitus (Celermajer *et al.*, 1994). Endothelial dysfunction occurs early and can even be detected in children with risk factors or a family history of premature coronary artery disease (CAD; Sorensen *et al.*, 1994; Clarkson *et al.*, 1997), suggesting that it is an early step in the disease process. In general the degree of endothelial dysfunction correlates with the number and severity of coronary risk factors (Vita *et al.*, 1990). Egashira and colleagues have described an association between endothelial dysfunction and an ischaemic syndrome due to microvascular endothelial dysfunction (manifested by a poor coronary blood flow response to intracoronary ACh) (Egashira *et al.*, 1996). Patients without haemodynamically significant epicardial artery stenoses but with evidence of exercise induced myocardial ischaemia (seen as a thallium perfusion defect) often show marked endothelial vasodilator dysfunction at the level of the microcirculation (Zeiher *et al.*, 1995). While endothelial dysfunction (and by association impaired NO

bioavailability) is highly predictive of vascular events (Schachinger *et al.*, 2000), this is not definitive proof of a causal relationship between the two. Further clarification of this relies on our understanding of the fundamental mechanisms underlying the loss of NO bioavailability, namely reduced synthesis and increased oxidative inactivation by reactive oxygen intermediates.

1.2 Animal models of cardiovascular disease

Impaired endothelium mediated vasorelaxation and reduced NO bioavailability can be demonstrated in animal models of cardiovascular disease including atherosclerosis, hypertension, and heart failure. NO inhibits platelet reactivity, VSMC proliferation, and monocyte adhesion, all of which contribute to the development of atherosclerosis (Cooke and Dzau, 1996). NO is a potent inhibitor of the expression of chemokines (such as monocyte chemotactic protein) and adhesion molecules (such as vascular cell adhesion molecule) that play a critical role in monocyte adhesion and infiltration (Tsao *et al.*, 1996; De Caterina *et al.*, 1995). The chronic administration of NOS inhibitors, either orally or subcutaneously, accelerates atherogenesis in animal models (Naruse *et al.*, 1994; Cayatte *et al.*, 1994). In hypercholesterolaemic rabbits, the chronic administration of arginine restores vascular NO activity, diminishes atheroma formation, and also induces the regression of preexisting lesions despite continuation of a high fat diet (Cooke *et al.*, 1992; Tsao *et al.*, 1994). Apolipoprotein E (ApoE)/eNOS double knockout mice demonstrate enhanced atherosclerosis compared to ApoE knockout controls (Kuhlencordt *et al.*, 2001). These findings strongly suggest that NO normally prevents the development of atherosclerosis.

In addition eNOS knockout mice are hypertensive, show exaggerated smooth muscle cell proliferative responses to injury, and develop aneurysms (Huang *et al.*, 1995; Shesely *et al.*, 1996). Gene transfer of eNOS and nNOS to carotid arteries *in vivo* attenuates neointima formation following balloon injury (Von Der Leyen *et al.*, 1995; Channon *et al.*, 1998). In humans, associations between polymorphisms of the eNOS gene, reduced enzyme activity and increased cardiovascular risk are strong genetic indicators of the role of NO deficiency in disease (Hingorani *et al.*, 1999).

1.3 Arginine paradox

The beneficial effects of arginine on endothelial function are controversial and are attributed to increased NO formation. The administration of arginine improves endothelium-dependent vasodilatation in cholesterol fed rabbits, in human subjects with hypercholesterolaemia, and in atheromatous human coronary arteries (Cooke *et al.*, 1992; Creager *et al.*, 1992; Drexler *et al.*, 1991; Boger *et al.*, 1997; Tousoulis *et al.*, 1997). The molecular explanation for these findings is unclear and some studies have failed to confirm these effects (Blum *et al.*, 2000). Any proposed mechanism must also explain the following paradox, known as the arginine paradox. Physiological arginine concentrations (rarely below 200μM) are present in apparent excess of the Km of NOS (approx 5μM), implying that arginine availability should not be rate limiting for NOS. In other words, the addition of arginine would in theory not be expected to influence NO formation *in vivo*. One explanation for the paradox might be that exogenous arginine produces biological effects independent to NO formation. L-arginine can, for example, stimulate endogenous insulin production that in turn causes vasodilatation (Giugliano *et al.*, 1997). Alternatively, Km

measurements for NOS *in vitro* may not reflect true functional Km values *in vivo*. Arginine concentrations within the cell may not be homogeneous due to compartmentalisation and local concentrations seen by NOS may not resemble average intracellular measurements. Caveolar complexes between the y+ (CAT1) arginine transporter and eNOS may provide a mechanism for the directed delivery of arginine to eNOS (Bogle *et al.*, 1995; McDonald *et al.*, 1997). Endogenous inhibitors of NOS, such as ADMA, may alter substrate availability (Vallance *et al.*, 1992).

1.4 Asymmetric methylarginines

Methylated arginine analogues, or methylarginines, are continuously formed within the intracellular compartment and can be detected in animal and human urine, plasma and tissue. Of these, the asymmetric methylarginines, ADMA and L-NMMA, inhibit all three NOS isoforms with comparable potencies *in vitro* and *in vivo*, in animals and in man. The IC50 for L-NMMA on all three NOS isoforms lies between 2 and 5μM. The IC50 for ADMA also lies between 2 and 5μM (Vallance *et al.*, 1992; Faraci *et al.*, 1995). Concentrations of ADMA *in vivo* are many times greater than those of L-NMMA in most published studies; ADMA has thus come to be regarded as the more important endogenous NOS inhibitor. The inhibitory effects of asymmetric methylarginines appear to be competitive in that they are reversible at least in part by the addition of arginine (Vallance *et al.*, 1992). The stoichiometry of this reaction however is far in excess of 1:1. This and other data suggests that asymmetric methylarginines may have additional non-competitive effects on NOS that are less easily reversed (Olken and Marletta, 1993; Tsikas *et al.*, 2000). Symmetric dimethylarginine (SDMA), a third methylarginine detected in animals and humans,

has no known biological function and does not inhibit NOS activity (Vallance *et al.*, 1992).

Asymmetric methylarginines may alter NOS function via other mechanisms although the relative importance of these *in vivo* is unclear. L-NMMA, ADMA and SDMA compete with each other and arginine for entry into cells via the y+ (CAT1) arginine transporter. Since the y+ (CAT1) arginine transporter lies in close proximity to NOS in caveolae, this arrangement may influence local substrate and inhibitor concentrations in the immediate vicinity of NOS (Bogle *et al.*, 1995; McDonald *et al.*, 1997). That purified NOS generates superoxide in preference to NO under some circumstances is supported by the observation that ADMA can increase superoxide formation by purified NOS and endothelial cells *in vitro* (Boger *et al.*, 2000). It is also possible that asymmetric methylarginines may regulate arginine handling enzymes other than NOS; NO synthesis represents about 1% of total arginine metabolism.

Asymmetric methylarginines may account at least in part for the arginine paradox for reasons described above. They inhibit NO synthesis and may potentiate superoxide formation, two of the key mechanisms underlying endothelial dysfunction (Vallance *et al.*, 1992; Boger *et al.*, 2000). Associations between plasma ADMA levels and cardiovascular risk factors as well as cardiovascular disorders have been described recently. These data suggest that ADMA may be a mediator, rather than a marker, of endothelial dysfunction and impaired NO synthesis in cardiovascular disease.

1.5 Biological and Clinical Significance of ADMA

Free ADMA concentrations in normal plasma range between 500nM and 1μM and are concentrated by a factor of 5 to 10 in cells (Vallance *et al.*, 1992; Azuma *et al.*, 1995). The normal 24 hour urinary excretion of ADMA is approximately 65 μmol/24h in humans (Vallance *et al.*, 1992). Raised urinary ADMA levels were first described in patients with muscular dystrophy and regarded as a marker of increased protein turnover in these patients (Lou, 1979). In 1992, Vallance and colleagues described the presence of ADMA in human plasma, and demonstrated that ADMA inhibits NOS as well as endothelium dependent vasorelaxation in the human forearm circulation *in vivo*. The discovery that plasma ADMA levels were raised in patients with renal failure led to speculation that the accumulation of endogenous ADMA might result in impaired NO synthesis *in vivo*, hypertension and increase cardiovascular risk in these patients (Vallance *et al.*, 1992). ADMA levels normalise in these patients following dialysis and these changes are associated with an improvement in endothelial function (MacAllister *et al.*, 1996b). These findings suggested that ADMA may be an important determinant of cardiovascular risk.

Many studies have now shown an association between raised ADMA levels and human cardiovascular disease or coronary risk factors in the absence of renal impairment. Healthy but hypercholesterolaemic individuals have an endothelial vasodilator dysfunction associated with a doubling of plasma ADMA levels and reduced NO generation (Boger *et al.*, 1998). Endothelial dysfunction in hypercholesterolaemia correlates better with ADMA than with LDL cholesterol levels and can be corrected by arginine supplementation (Boger *et al.*, 1998). Raised ADMA levels have also been described in patients with other coronary risk factors such as

insulin resistance, diabetes and hyperhomocysteinaemia (Lin *et al.*, 2002; Stuhlinger *et al.*, 2001). These observations indicate that ADMA may act as a mediator of endothelial dysfunction under the influence of more traditional risk factors.

Raised plasma ADMA levels have been described in adults and children with hypertension (Matsuoka *et al.*, 1997; Goonasekera *et al.*, 2000). In salt sensitive patients with essential hypertension, administration of a high salt diet increases ADMA levels and blood pressure as well as reduces NO synthesis; the opposite changes are seen following a low salt intake (Fujiwara *et al.*, 2000). These findings suggest that ADMA levels may be dynamically regulated. Plasma ADMA levels are elevated in patients with peripheral arterial disease and can be a strong independent predictor of early atherosclerosis in asymptomatic subjects as determined by carotid intima-media thickness (Miyazaki *et al.*, 1999). Valkonen and colleagues have reported a strong positive association between ADMA concentrations and coronary risk in non-smoking men in Finland (Valkonen *et al.*, 2001). In a recent analysis of a large group of patients with end stage renal failure by Zoccali and colleagues, plasma ADMA concentrations emerged as second only to age as a predictor of overall mortality and cardiovascular risk (Zoccali *et al.*, 2001). A further study in the same patient group demonstrated an inverse relationship between ADMA levels and left ventricular (LV) ejection fraction; in a multivariate analysis which included LV end diastolic volume and heart rate, ADMA was shown to be an independent and strong predictor of LV ejection fraction in patients with renal impairment. ADMA also appears to be a strong and independent predictor of left ventricular (LV) mass and concentric LV hypertrophy (Zoccali *et al.*, 2002).

ADMA levels are elevated significantly in patients with hemorrhagic shock and stroke (Aneman *et al.*, 1994; Yoo *et al.*, 2001). Animal studies have shown that ADMA levels accumulate in the rabbit proximal urethra following ischaemia due to iliac artery occlusion (Masuda *et al.*, 2001; Masuda *et al.*, 2003). ADMA inhibits angiogenesis in an *in vivo* disk model of angiogenesis (Jang *et al.*, 2000) and may account for impaired angiogenesis in animals with hypercholesterolaemia (Duan *et al.*, 2000). Endothelial cells regenerating after rabbit carotid arterial injury have impaired endothelial vasodilator function in association with increased intracellular ADMA levels indicating that ADMA may impair NO synthesis following percutaneous coronary interventions (Azuma *et al.*, 1995). These changes are exaggerated following nicotine exposure and alloxan-induced hyperglycaemia in these animals (Hamasaki *et al.*, 1997; Masuda *et al.*, 1999). ADMA levels are also significantly raised in patients with chronic heart failure (Usui *et al.*, 1998), rats with heart failure induced by coronary artery ligation (Feng *et al.*, 1998), and dogs with heart failure induced by rapid pacing (Ohnishi *et al.*, 2002).

These associations indicate a possible role for ADMA in the pathophysiology of endothelial dysfunction and associated cardiovascular diseases. ADMA levels may also help to explain the arginine paradox described above. Although abnormal plasma ADMA levels rarely exceed 10μM, these 'spillover' levels could be associated with much greater intracellular levels and thus alter NO synthesis to an important degree. In patients with septic shock, circulating L-NMMA concentrations of about 5μM following exogenous L-NMMA infusion increase systemic vascular resistance and blood pressure by >70% and 15% respectively (Petros *et al.*, 1991). Increasing plasma ADMA concentrations to approximately 10 μM in animal models increases blood pressure by approximately 15% (Vallance *et al.*, 1992). Chronic increases to these

levels may have even more profound effects. It may also be that plasma ADMA levels are not truly representative of intracellular ADMA levels, which in turn reflect a dynamic balance between ongoing protein methylation, protein turnover and ADMA metabolism. An understanding of this balance is fundamental to the study of a relationship between ADMA and human disease.

1.6 Methylarginine formation

1.6.1 Protein arginine methyltransferases

Methylarginines are continuously formed in all cells by the methylation and subsequent turnover of proteins. The transfer of methyl groups (from S-adenosyl-L-methionine) to arginine residues within proteins is a fundamental control of protein function and is catalysed by a family of protein arginine methyltransferases (PRMTs; reviewed in Aletta *et al.*, 1998). Type I PRMTs (PRMT 1, 3, 4 and 6, of which PRMT 1 is by far the most abundant) specifically catalyse the formation of L-NMMA and ADMA in a number of proteins including RNA binding and transporting proteins, nuclear matrix proteins, histones, heat shock proteins and myosin. Type II PRMT (PRMT 5) forms part of a methylosome complex and generates LNMMA and SDMA residues in myelin basic protein. Consistent with these differences is the observation by Kakimoto and colleagues that while SDMA is the predominant methylarginine in protein extracts from white matter of human and bovine brains, ADMA predominates in all other parts of the brain as well as in all other tissues (Kakimoto *et al.*, 1975). The ADMA content in intact rat proteins lies roughly between 1 and 2 umol/g of intact protein (Kakimoto *et al.*, 1975). Probably more than half of cellular proteins are methylated in this way and tend to accumulate in the nucleus (Miyake and Kakimoto,

1976). The rate of protein methylation appears to depend on the rate of protein synthesis perhaps because methylation occurs soon after synthesis (Miyake and Kakimoto, 1976). The regulation of PRMT expression, by factors such as nerve growth factor (Cimato *et al.*, 2002), is also likely to influence the rate and nature of protein methylation.

1.6.2 Protein turnover

Since there is no evidence that the demethylation of intact proteins can take place, any reversal of protein methylation must depend on the breakdown of these proteins. Protein breakdown lies in balance with the rate of protein synthesis to maintain the net protein content of cells. Between 1 and 2% of total body protein is turned over daily. Adults catabolise approximately 300g protein per day, the bulk of this (approximately 30%) resulting from muscle turnover. In critically ill patients, approximately 20% of body protein can be lost over 3 weeks (of which 70% is derived from skeletal muscle; Cuthbertson, 1964). Protein degradation in mammalian cells is reliant on a widely distributed ATP dependent ubiquitin-proteasome pathway; major changes in cell morphology and protein expression are associated with increased protein turnover and proteasome activity (Wojcik and Wilk, 1999). The ubiquitin-proteasome pathway also becomes more active during catabolic states associated with enhanced proteolysis (including denervation, hyperthyroidism and sepsis). Matrix metalloproteinases are upregulated during vascular remodelling, angiogenesis, atherogenesis, heart failure and following percutaneous coronary interventions (Dollery *et al.*, 1995; Libby and Schonbeck, 2001; Libby, 2001; Feldman *et al.*, 2001). The upregulation of lysosomal proteases (cathepsins) has been described in atherosclerotic lesions (Libby, 2001).

Increased protein breakdown seen in these and other conditions may increase methylarginine formation. Given that protein methylation is related to the rate of protein synthesis, changes in methylarginine formation may be magnified further in the setting of increased protein turnover. The irony is that the preservation and even upregulation of NOS function is often necessary in these situations. Mechanisms to prevent the accumulation of asymmetric methylarginines may therefore be particularly important in these situations.

1.7 Methylarginine metabolism

1.7.1 Metabolism of methylarginines

As indicated above, the continuous generation of asymmetric methylarginines poses a potential and constant threat to NO formation. This threat may be magnified in physiological and disease states where increased protein methylation and turnover coincide with a need for NOS upregulation. The metabolism of methylarginines by cultured endothelial cells and human blood vessels has been clearly demonstrated (Hecker *et al.*, 1990; MacAllister *et al.*, 1994). Evidence from animal studies suggest that asymmetric methylarginines are metabolised extensively *in vivo* although there is also some evidence to suggest that this may vary across different species. The same studies suggest that SDMA, which is not a NOS inhibitor, is not metabolized to the same degree.

McDermott showed in 1976 that urinary SDMA concentrations are 30 times higher than those of ADMA in rabbits even though plasma ADMA levels are higher than those of SDMA (McDermott, 1976). The urinary recovery of intravenously injected

ADMA (5%) was significantly lower than that of SDMA (66%) in rabbits, suggesting that while SDMA is largely excreted unmetabolised in the urine ADMA is metabolised to a significant degree *in vivo*. Following the intravenous injection of radioactively labelled (^{14}C) L-NMMA into rabbits, only 33% of radioactivity was recovered in the urine after 8 days and of this only 1.5% represented unmetabolised ^{14}C-L-NMMA (McDermott, 1976).

Many years later, Ogawa and colleagues demonstrated that following the intraperitoneal injection of ^{14}C-ADMA in rats, only 13% of the radioactivity was recovered in the urine after 12 hours (Ogawa *et al.*, 1987). In contrast, the recovery of total radioactivity following ^{14}C-SDMA injection was 84%. Amounts of expired radioactivity ($^{14}CO2$) were six times higher in animals receiving ^{14}C-ADMA rather than ^{14}C-SDMA, suggesting that the metabolic fate of ADMA is more complex. Radioactivity retained in a range of tissues following ^{14}C-ADMA injection was mostly in the form of ^{14}C-citrulline. Much smaller amounts of labeled ornithine and arginine were also present, suggesting that labeled citrulline was more likely to have been derived from ^{14}C-ADMA directly (rather than via ornithine) before being further metabolized via a pathway related to the urea cycle. There was also evidence for the incorporation of radioactivity into proteins (as labelled protein-bound arginine only) following ^{14}C-ADMA, but not ^{14}C-SDMA, injection. This demonstrated that ADMA has the potential to be recycled to arginine for protein synthesis and is consistent with *in vitro* studies demonstrating the recycling of methylarginines to arginine in arginine-depleted endothelial cells (Hecker *et al.*, 1990). More recently, Schwartz and colleagues have shown that following a single intravenous infusion of ^{14}C-L-NMMA, exhaled $^{14}CO2$ accounted for 53% and 23% of the administered radioactivity lost after seven days in rats and dogs respectively (Schwartz *et al.*, 1997). Although technical

differences in sample collection may account for these differences, it is likely that differences in asymmetric methylarginine metabolism exist across animal species. In summary, animal studies have indicated that the extensive metabolism of asymmetric methylarginines, but not symmetric methylarginines, takes place *in vivo*.

1.7.2 Dimethylarginine Dimethylaminohydrolase

The findings described above set the scene for the discovery of a new enzyme dimethylarginine dimethylaminohydrolase (DDAH) in rat tissues by Ogawa and colleagues in 1989. DDAH metabolises the asymmetric methylarginines ADMA and L-NMMA to form citrulline and dimethylamine or monomethylamine respectively and appears to do so in the absence of essential cofactors. DDAH does not appear to metabolise SDMA. The crystal structure of DDAH suggests that it belongs to a superfamily of arginine-modifying enzymes including arginine:glycine amidinotransferase and arginine deiminase, and also that it exists as a dimer with two active sites within each dimer functioning independently (Murray-Rust *et al.*, 2001). Two human isoforms of DDAH, DDAH1 and DDAH2, have been identified and are virtually similar in terms of their activity and substrate specificity (Leiper *et al.*, 1999). The human genes encoding DDAH1 and DDAH2 lie on chromosomes 1p22 and 6p21.3 respectively (Tran *et al.*, 2000). The tissue distribution of DDAH1 corresponds approximately to that of nNOS while DDAH2 appears to be expressed in tissues expressing predominantly eNOS (Leiper *et al.*, 1999). This is suggestive of an isoform specific mechanism for the regulation of NOS by asymmetric methylarginines. The observation that DDAH is highly conserved across a range of species also supports its functional importance. Microbial DDAH genes appear in

operons involved in arginine metabolism, implying an evolutionary link to the regulation of arginine utilizing enzymes and further suggesting an important role for DDAH (Santa Maria *et al.*, 1999).

1.7.3 Biological and Clinical Significance of DDAH

The regulation of NOS by endogenous asymmetric methylarginines, and in particular ADMA, has been discussed at length. The metabolism of asymmetric methylarginines by DDAH in animals *in vivo* and in human tissues and endothelial cells *in vitro* raises the possibility that DDAH may indirectly regulate NOS. The pharmacological inhibition of DDAH increases ADMA accumulation in cell culture medium (but has no effect on SDMA levels), impairs NO synthesis by cultured cells, and causes the endothelium dependent contraction of rat aortic rings in organ baths studies (MacAllister *et al.*, 1996). These effects can be reversed by exogenous arginine supporting the view that a balance between arginine and ADMA levels may determine NOS activity. DDAH may regulate or facilitate NO formation by preventing or limiting the accumulation of ADMA. This is supported by the spatial relationship between the expression of NOS and DDAH isoforms described earlier (Leiper *et al.*, 1999).

Evidence for the metabolism of ADMA by DDAH in humans *in vivo* is limited. Since DDAH metabolises ADMA but not SDMA, alterations in the ADMA:SDMA ratio may reflect changes in DDAH activity. In chronic renal failure patients, SDMA levels accumulate to a greater extent (8-fold) than ADMA (3-fold) demonstrating that significant ADMA metabolism probably takes place in humans *in vivo* (Vallance *et al.*, 1992; MacAllister *et al.*, 1996b). In other disease states, levels of ADMA but not

SDMA are raised. This suggests that impaired DDAH activity might account for raised ADMA levels and hence impaired NOS function. A reduction in purified and/or tissue DDAH activity by oxidative stress, nitrosylation, hyperglycaemia, or homocysteine is associated with a rise in ADMA elaboration *in vitro* (Ito *et al.*, 1999; Leiper *et al.*, 2002; Lin *et al.*, 2002; Stuhlinger *et al.*, 2001). Laussmann and colleagues have recently demonstrated the upregulation of DDAH1 mRNA and protein expression by over 300% in regions of low myocardial blood flow in intact dog hearts compared to regions of high flow (Laussmann *et al.*, 2002). Changes in DDAH1 expression have also been described following nerve injury (and in association with coordinate changes in nNOS expression; Nakagomi *et al.*, 1999) as well as in patients with Alzheimer disease (Smith *et al.*, 1998). These findings suggest that dynamic changes in DDAH expression and activity may take place in response to physiological and pathological stimuli, and that these changes may in turn produce dynamic changes in ADMA levels and NO formation.

There are some theoretical concerns about the ability of DDAH to metabolise ADMA *in vivo*. While intracellular concentrations of ADMA may be in the order of 10μM, the Km of DDAH is much higher (360μM and 510μM for purified DDAH1 and DDAH2 respectively). It might therefore be argued that DDAH cannot metabolise ADMA to any significant degree *in vivo* even though animal studies described above have shown that this is not the case. One explanation may be that the functional Km of DDAH *in vivo* is different to that seen with purified enzyme preparations, as in the case of NOS. The Km for DDAH in tissues is approximately 140μM and even this may represent an overestimate. Dimerisation or post-translational modification of DDAH may alter the Km values (Murray-Rust *et al.*, 2001). It is also possible that high local concentrations of ADMA are reached in some circumstances or in specific

intracellular compartments in relation to sites of arginine transport or protein breakdown. Thirdly it is possible that DDAH has functions unrelated to ADMA metabolism. It has recently been shown that DDAH can associate with neurofibromin and regulate its phosphorylation by cAMP-dependent protein kinase (Tokuo *et al.*, 2001). Regardless of these theoretical concerns, it is clear that DDAH is highly conserved, expressed in a variety of tissues, and regulated during development and possibly during disease. It is also clear that the metabolism of ADMA to citrulline takes place to a significant degree in animals *in vivo*. The same is likely to be true in humans but this has not been proven to date.

1.8 Summary

Growing evidence suggests that NO is an important mediator of cardiovascular, neurological, and immune function. Endothelial dysfunction, synonymous with impaired NO synthesis, is associated with a range of cardiovascular diseases and may represent an early step in their development. An endogenous inhibitor of NOS, ADMA, is produced by cells continuously and can inhibit all three isoforms of NOS. Although ADMA levels may to some extent be dependent on the cycle of protein synthesis, methylation and breakdown, an important determinant of ADMA levels appears to be its metabolism by DDAHs. Organ bath data suggest that DDAHs may regulate NOS indirectly (MacAllister *et al.*, 1996).

The role of ADMA metabolism by DDAH in NOS regulation may be more important in those situations associated with increased protein turnover. Cellular differentiation and tissue injury are two situations in which an increase in protein turnover coincides with an important role for NO signalling. A vitamin A derivative, all-*trans*-retinoic

acid (atRA), regulates tissue development and remodelling and may act via NO (Ghigo *et al.*, 1998). atRA also has significant effects on the cardiovascular system which may be endothelium dependent. We hypothesised that atRA might regulate endothelial NO synthesis and investigated the possible role of ADMA metabolism by DDAH. NO signalling also plays a critical role in the response to tissue injury secondary to ischaemia. Studies described in this thesis tested the hypothesis that the ADMA/DDAH pathway might play a role in the response to ischaemia using an *in vivo* mouse model of unilateral hindlimb ischaemia.

Raised plasma ADMA levels have been described in association with endothelial dysfunction, implying that ADMA may be a circulating marker if not an intracellular mediator of endothelial dysfunction. Cardiovascular diseases associated with endothelial dysfunction are also associated with raised plasma ADMA levels. Recent studies show that ADMA may be a very strong and independent predictor of atherosclerosis and LV dysfunction in patients. Further studies described in this thesis tested the hypothesis that ADMA regulates cardiovascular function in humans *in vivo* and that these effects might be consistent with those abnormalities seen in human disease. The hypothesis that ADMA is metabolised by DDAHs in humans *in vivo* was also tested.

In cardiovascular disease states, impaired DDAH activity may represent a fundamental cause of ADMA accumulation and endothelial dysfunction. Strategies that target DDAH and restore NO synthesis by reducing ADMA levels may have therapeutic benefit. This highlights the importance of studying the cardiovascular effects of ADMA and its metabolism by DDAH in health and human disease.

Chapter Two: Methods

2.1 Molecular and Cellular Biology

2.1.1 Polymerase Chain Reaction

DNA sequences were amplified by Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988). Typically, PCR reactions contained oligonucleotides at 1pmol/μL (Sigma-Genosys), 0.1mM dNTPs (Gibco BRL), 1.5mM $MgCl_2$, Taq DNA polymerase 0.2U and 1X Taq buffer (all from Gibco BRL) in a final volume of 50μL. Amplification conditions were 94°C for 5 minutes; 30 cycles of 94°C (30s), 55°C (30s), 72°C (30s); and 72°C for 5 min, unless otherwise stated.

In the transgenic mouse study, screening of genomic DNA samples was done by PCR using transgene specific oligonucleotide primers (5'- AGCACCAGCTCTACGTG-3' and 3'- GCCCTTTGTTGGGGATATT-5').

2.1.2 Reverse Transcription

Reverse transcription (RT) was performed using Ready-To-Go™ You-Prime first-Strand Beads (Amersham) which utilize Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase to generate first strand cDNA. Typically, 1μl RNA was heated with 29μl of diethyl pyrocarbonate (DEPC, Sigma) treated water (RNAse free water), to 65°C for 10 minutes and chilled on ice for 2 minutes. The reaction mixture was transferred to a fresh RT-PCR tube containing the RT-beads and 0.2μl of reverse oligonucleotide primer added. After incubation at room temperature for 1 minute, tubes were mixed by gentle vortex followed by centrifugation and incubated at 37°C for 1 hour.

Probes for northern blotting were produced by reverse transcription followed by PCR (RT-PCR). A probe for mouse DDAH2 was produced by RT-PCR of sEnd.1 cell total RNA using oligonucleotides mDDAH II.1 and mDDAH II.2 (complementary to base pairs -224 to -205 and 346 to 324 of mouse DDAH II cDNA respectively). A probe for mouse eNOS was produced in a similar way using oligonucleotides mENOS1 and mENOS2 (complementary to base pairs 3310 to 3328 and 3621 to 3602 of mouse eNOS cDNA respectively).

2.1.3 Agarose gel electrophoresis

Products of PCR or restriction digests were analysed by agarose gel electrophoresis. Agarose (0.6-1.5% w/v) (Gibco BRL) was made up in 1X TBE (10X TBE from Gibco BRL) and melted by heating to >60°C. Ethidium bromide (Sigma) was added to a final concentration of 0.2mg/mL. Gel loading solution (6X) (Sigma) was added to DNA samples, typically 2μL of gel loading solution was added to 10μL sample, and loaded onto a gel. Samples were electrophoresed in 1X TBE at 100V for ~30 min. DNA fragments were visualised using a UV transilluminator and photographs taken.

2.1.4 Cell Culture

sEnd.1 cells (murine skin endothelioma cells) were originally isolated from murine haemangiomas induced by injection of a neomycin resistant retroviral vector expressing the middle T oncogene (Williams *et al.*, 1989). sEnd.1 have been shown to retain endothelial cell morphology and express specific endothelial cell markers.

Under normal conditions, in culture, sEnd.1 cells express eNOS and make low but detectable levels of NO (nitrite levels measured by Greiss assay). sEnd.1cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). ECV304 cells were cultured in M199 medium containing 10% FCS. Primary porcine aortic endothelial cells (PAEC; single donor; passage 2 to 7) were prepared by the collagenase treatment of porcine aorta and cultured in DMEM containing 10% FCS. SV40 transfected human umbilical vein endothelial cells (SGHEC-7) were cultured in medium M199:RPMI 1640 in a ratio of 1:1 supplemented with 2.5μg/ml endothelial cell growth supplement, 0.09mg/mL heparin, 2.5% FCS and 2.5% newborn calf serum. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and were routinely passaged every 7 days using trypsin/EDTA.

2.1.5 RNA isolation

Total RNA was isolated from confluent cell monolayers grown in 6-well tissue culture plates (approximately 10^6 cells/well at the time of treatment) using TRIzol (Gibco-BRL) according to the manufacturer's instructions. TRIzol is a mono-phasic solution of phenol and guanidine isothiocyanate and maintains the integrity of RNA while disrupting cells and dissolving cell components. Cell monolayers were lysed and harvested by adding 1mL of TRIzol reagent to each well. 0.2mL of chloroform was then added and the tubes shaken vigorously for 15s before centrifugation at 14,000rpm for 15min at 2-8°C to separate the organic and aqueous phases. RNA in the upper aqueous phase was transferred to fresh tubes and precipitated by mixing

with 0.5mL isopropanol and centrifuging at 14,000rpm for 10min at 2-8°C. After washing with 75% ethanol, RNA pellets were resuspended in 10μL DEPC (Sigma) - treated water or 20μL of a solution of RNA loading buffer (Sigma) mixed with DEPC-treated water in a ratio of 2:1.

2.1.6 Northern Blotting

Total cellular RNA isolated using TRIzol (Sigma) was electrophoresed through 1% (w/v) agarose-formaldehyde gels prepared as follows. 1.5g of agarose (Gibco BRL) was melted in 127mL of DEPC-treated water and allowed to cool to 60°C. In a fume hood, 15mL of 10X MOPS buffer (Sigma) and 8mL of 37% formaldehyde (BDH) was added. The gel solution was mixed, poured and allowed to set in the fume hood. RNA loading buffer containing ethidium bromide (Sigma) was added to RNA samples in a ratio of 2:1 of buffer to sample. Samples were denatured at 60°C for 15 min prior to loading and electrophoresed in 1X MOPS buffer at 100V for ~1.5h. Gels were visualised on a UV transilluminator as described before and blotted immediately.

Transfer of RNA to Hybond-N+ positively charged nylon membranes (Amersham) was performed by capillary blotting. A tray was filled with blotting buffer (20X SSC) and a platform made which could be covered with a wick made from Whatman 3MM paper saturated with blotting buffer. The RNA gel was placed on the wick without trapping air bubbles beneath it. A sheet of Hybond-N+ membrane the exact size of the gel was placed on top of the gel without trapping air bubbles beneath. The membrane was surrounded with cling film to prevent the blotting buffer being

absorbed into the paper towels to be placed above. Three sheets of 3MM paper cut to size and saturated with blotting buffer were placed on top of the membrane. A stack of absorbent paper towels (about 5cm high) was then placed on top of the 3MM paper. Transfer was allowed to proceed for ~16 hours. Following transfer, RNA was cross-linked to the membrane by UV light in a UV Stratalinker (Stratagene).

A murine DDAH I probe was obtained from an IMAGE clone (no. 732946, accession no. AA403666) which contains an insert identical to base pairs 371 to 870 of mouse DDAH I cDNA. cDNA probes for mouse DDAH2 and eNOS were produced by RT-PCR as described above. Probes were then labelled using a Random Primed DNA Labelling Kit (Roche). Typically, 2μL of the gel-purified product was added to a labelling reaction containing dATP, dGTP, dTTP at 25μM each, 1X hexanucleotide reaction mixture, 2U Klenow enzyme and 2μL (0.925MBq) [^{32}P]dCTP in a total volume of 20μL. This labelling reaction was incubated at 37°C for 30 min.

Separation of the DNA probe from unincorporated ^{32}P-labelled nucleotides was performed using NICK columns (Pharmacia Biotech) according to manufacturers instructions. Briefly, columns containing Sephadex G-50 DNA Grade were equilibrated with TE buffer (10mM Tris-HCL (pH8), 1mM EDTA) prior to addition of the labelling reaction. Three 400μL-eluates in TE buffer were then eluted from the column. The second fraction containing labeled DNA was used in hybridisation experiments. The remaining fractions containing unincorporated radioactivity were discarded. The labelled probe was denatured at 100°C for 10 min prior to hybridisation.

Northern hybridisation experiments were carried out using ExpressHyb Hybridisation Solution (Clontech Laboratories, Inc.) as recommended by the manufacturer. The

ExpressHyb Hybridisation Solution is optimised for Southern and northern analyses on positively charged nylon membranes and requires only 1-2h of hybridisation. The solution was pre-warmed to 60°C (Southern) or 68°C (northern) prior to addition of 5mL to a hybridisation tube containing the membrane and incubated for 30 min at the appropriate temperature with continuous shaking in a Techne Hybridiser HB-1. During this prehybridisation period, the radioactively labelled probe was denatured by heating to 100°C for 10 min and chilled on ice. The prehybridisation solution was then replaced with 10mL fresh hybridisation solution containing the denatured probe. The membrane was then incubated for 1h with continuous shaking at 60°C (Southern) or 68°C (northern). Following hybridisation, the blots were washed for 1h in Wash Solution 1 (2X SSC, 0.05% SDS) at room temperature with continuous shaking. The solution was replaced 3 times during this period. This was repeated with Wash Solution 2 (0.1X SSC, 0.1% SDS) at 50°C. Blots were then wrapped in Saranwrap and exposed to a phosphorimager screen overnight (DDAH1, DDAH2, eNOS, nNOS, and iNOS probes) or 30 min (β–actin probe) for northern blots. Signals on northern blots were detected and visualised on a Fuji BAS1000 phosphorimager and quantified using Tina 2.08e software.

2.1.7 Western Blotting

In cell culture experiments, cells were grown to confluence in 75cm^2 flasks and lysed with 0.1% SDS. In the animal and tissue studies, tissues were homogenized on ice in 3 volumes of buffer containing 0.5% sodium deoxycholate and 0.1% SDS. Homogenates were centrifuged at 3000G for 20 min and supernatants stored on ice for immediate use. Protein concentrations were either determined using the method of

Lowry et al (Lowry *et al.*, 1951) or using a Bio-Rad protein assay kit with bovine serum albumin standards (see below). 50-100μg of protein samples were then separated on a 12% SDS-polyacrylamide gel under reducing conditions and electrophoretically transferred to a methanol presoaked nitrocellulose membrane (Hybond P, Amersham). Once transferred, the membrane was blocked in 5% non fat milk (w/v) made up in phosphate buffered saline solution containing 1% polyoxyethylene-sorbitan monolaurate (Tween 20, Sigma) (PBST) to minimise non-specific binding and subsequently exposed to a primary antibody specific for the protein of interest, diluted in PBST or PBST containing 5% milk powder (as specified) for either 1-2 hours at room temperature and pressure or overnight at 4°C. Following this incubation period the membrane was thoroughly washed in 6 x 15 minute washes using PBST before incubation with a secondary antibody linked to horseradish peroxidase (HRP) for 1-2 hours. Following incubation with the secondary antibody, the membrane was again thoroughly washed in 6 x 15 minute washes of PBST and immunoreactivity visualised using a using an ECL detection kit that detects HRP (Amersham, Buckinghamshire, UK). Quantification was performed by densitometry (NIH *Image*, NIH).

In the cell culture experiments, membranes were probed with a polyclonal antibody (Ab) specific for DDAH2. The DDAH2 polyclonal Ab was raised in rabbit against a $DDAH2_{241-255}$ peptide. In the animal studies, membranes were probed with monoclonal antibodies (Ab) specific for human DDAH or rat DDAH (supplied by M Kimoto, Okayama Prefectural University, Kuboki, Japan), nNOS, eNOS, iNOS (Transduction Laboratories, Lexington, KY), and VEGFR2 (Sigma, St Louis, MO). Immunoreactive bands were visualized with a horseradish peroxidase conjugated secondary Ab.

2.2 Biochemical Assays

2.2.1 Protein assay

Protein concentrations in cell lysates and tissue extracts were measured using the Bio-Rad protein assay, based on the Bradford colorimetric assay for measuring total protein concentration. Protein standards were prepared by serial dilutions of 10mg/mL BSA (bovine serum albumin). Protein samples (from cell or tissue samples) were diluted to ensure they were in the range of the standard curve. Bio-Rad reagent was added in a 1:5 dilution to all samples and standards in a 96 well plate, mixed and incubated for 5min. Absorbance at 595nm was measured in a SpectraMax 250 microplate spectrophotometer (Molecular Devices). All lysates were assayed in duplicate.

2.2.2 Griess assay

The final products of NO *in vivo* are nitrite and nitrate. NO production by sEnd.1 cells was determined by measuring nitrite accumulation in cell culture medium (Griess reaction). Standards up to 100μM were prepared by serial dilution of sodium nitrite in PBS. Samples were initially incubated with a mixture of nitrate reductase and lyophilised cofactors at room temperature for one hour to convert nitrate to nitrite. Equal volumes of Griess Reagent A (0.1% (w/v) naphthalethylenediamine HCl) and Griess Reagent B (1% (w/v) sulphanilamide in 5% (w/v) phosphoric acid) were mixed. 100μL of this mixture was then added to 100μL of sample, standard or blank, mixed and incubated at room temperature for 15min. Absorbance at 540nm was read

in a SpectraMax 250 microplate spectrophotometer (Molecular Devices). All samples were assayed in duplicate.

2.2.3 NOS Activity Assay

NOS activity of tissue homogenates was determined by a radiochemical assay measuring the rate of conversion of ^{3}H Arginine (Amersham, Buckinghamshire, UK) to ^{3}H-Citrulline. Tissues were mechanically homogenised in 0.1M potassium phosphate solution (pH7) on ice (3mL KPO_4/g tissue) and spun at 3000rpm for 20min at 4°C. 15µL of tissue supernatant was incubated with 0.1µCi ^{3}H-arginine, 2.5µM (cold) arginine, 1mM NADPH, 3µM tetrahydrobiopterin, 1µM FAD, 1µM FMN and 600µM CaCl2 in a total volume of 50µL for 30min at 37°C. The reaction was terminated by the addition of 500µL 1:1 Dowex-50W (Na form) and the supernatant was removed for detection of ^{3}H-Citrulline by liquid scintillation counting (Beckman, Fullerton, CA). NOS activity was determined from the difference between standard reactions and reactions containing 1mM NG-nitro-L-arginine methyl ester (L-NAME) and expressed as counts/µg protein/min.

2.2.4 ADMA and SDMA Measurements

Concentrations of ADMA and SDMA in conditioned medium and human plasma were measured by high performance liquid chromatography (HPLC) as described previously (Vallance *et al.*, 1992). Samples were loaded onto BondElut SCX columns and methylarginines eluted in 50% (v/v) ammonia in methanol which was then

evaporated to dryness at 130°C under nitrogen. Dried extract was then dissolved in water, loaded onto a CBA column and dimethylarginines again eluted in 10% ammonia/methanol before evaporation at 130°C under nitrogen. Dried extract was then redissolved in running buffer consisting of 0.025M orthophosphoric acid, 0.01 hexanesulphonic acid, and 1% (v/v) acetonitrile in distilled water, prior to injection onto an ODS C18 analytical HPLC column (Spherisorb, PhaseSep, UK). Flow rate was maintained at 1 ml/min and linked to a Beckman System Gold HPLC system. Absorbance was determined at 205nm. Peak area was calculated by computerised integration. Samples were spiked with a known amount of homoarginine as an internal standard to allow calculation of methylarginine concentrations.

Methylarginine concentrations in animal plasma and tissue were measured after precolumn derivatization with o-phthaldialdehyde (OPA), separation by HPLC and measurement using a fluorescence detector (excitation of 340nm and emission of 450nm and 455nm). The recovery rate for ADMA was 85% and the intra-sample variation was 7%. The detection limit of the assay was 0.1μM.

2.2.5 Creatinine Assay

Urinary creatinine levels (mg/dL) were measured using a method modified from that of Slot and developed by Sigma Diagnostics (Heinegard and Tiderstrom, 1973). Urine samples were diluted 10-fold. 1ml Alkaline Picrate solution was added to 100μl of sample/standard/blank and allowed to stand at room temperature for 10mins to form a yellow/orange colour. 110μl was removed and labelled as A. To the remaining 990μl, 30μl Acid Reagent (a mixture of sulphuric and acetic acid) was added and incubated

at room temperature for 5mins. This destroys colour derived from creatinine. A second 110µl aliquot was removed and labelled as B. Absorbance at 500nm was read in a SpectraMax 250 microplate spectrophotometer (Molecular Devices). Creatinine concentrations were calculated from the difference between A and B. All samples were assayed in duplicate.

2.2.6 Dimethylamine Assay

Dimethylamine levels (µM) were determined by the method of Beal and Bryan (1978). 200ul Reagent A (2g Ammonium Acetate and 20mg CuSO4.5H2O in 3ml water + 1g NaOH in 2.5ml water + 2ml NH4OH) and 600ul Reagent B (5% Carbon Disulphide solution in Benzene) were added to 400µl sample/standard/blank before agitation and the addition of 100µl 30% acetic acid. After further agitation, absorbance at 434nm was read in a SpectraMax 250 microplate spectrophotometer (Molecular Devices). All samples were assayed in duplicate.

2.2.7 DDAH Activity Assay

DDAH enzyme activity was assayed by determining citrulline formation from ADMA. Briefly, 50µL of tissue homogenate was incubated with 500µM ADMA and 100mM potassium phosphate buffer (pH 6.5) in a total volume of 100µl. The mixture was incubated at 37°C for 90 min and the reaction stopped by the addition of 10% trichloroacetic acid. Following the addition of 1:1 Dowex-50W (Na form), supernatant was removed for measurement of citrulline by the method of Prescott and

Jones (Prescott and Jones, 1969). Briefly, reagents A (0.8g diacetylmonoxime in 100mL 5% acetic acid) and B (0.5g antipyrine in 100mL 50% H2SO4) was mixed in the ratio 1:2. 500μL of this mixture was added to 500μL sample or standard, mixed, boiled at 87°C for 37min, and cooled on ice. Absorbance at 466nm was read in a SpectraMax 250 microplate spectrophotometer (Molecular Devices). Reactions kept at -20°C were used to obtain background values. These were subtracted from experimental data to determine DDAH activity (expressed as μmol citrulline/μg protein/min).

2.3 Mouse Studies

2.3.1 Animal Care

Transgenic mice overexpressing human DDAH I under the control of a human β-actin promoter were generated on a pure genetic background (C57BL/6J) were made by the Transgenic Research Facility at Stanford University. C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were also used for the breeding of transgenic mice and controls. All protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. Mice were maintained under controlled environmental conditions on a 12hr light/dark cycle and provided with standard chow and water *ad libitum*.

Genomic DNA was isolated from tail biopsies at 3 to 4 weeks of age using a DNeasy kit (Qiagen, Valencia, CA). Samples lysed overnight with proteinase K were loaded onto DNeasy columns before centrifugation. DNA bound to a silica gel-membrane

and was then eluted using a low-salt buffer. Screening of genomic DNA samples was done by polymerase chain reaction using transgene specific oligonucleotide primers as described above.

2.3.2 Measurement of Urinary Nitrogen Oxides

Mice were placed in metabolic chambers overnight for urine collection. Urine was collected in test tubes containing 100µl isopropyl alcohol submerged in ice water for the duration of the collection. Urine samples were then centrifuged at 4000rpm for 5mins and the supernatant was removed for storage at –80°C. Urinary nitrogen oxides were measured using the Griess reaction.

2.3.4 Non-invasive Blood Pressure Measurements

Systolic blood pressure was measured in conscious mice by tail plethysmography (Visitech 2000, Visitech Systems, Apex, NC) as previously described (Krege *et al.*, 1995; Figure 1). All mice were trained on alternate days for a period of three weeks in order that they become accustomed to the apparatus. Blood pressure measurements were then made on three separate days using a single tail cuff for all measurements. On each day, each mouse was allowed to rest on a heated plate for 15 minutes with its tails placed inside the tail cuff. The cuff was inflated and released once to prepare the mouse. Thereafter, ten consecutive systolic blood pressure measurements were made, recorded by a computer (Blood Pressure Analysis System, Visitech Software) and averaged to obtain a mean value for that day. Final values for each mouse were calculated as the mean measurement over three days.

Figure 1. Systolic blood pressures were measured in mice by tail plethysmography (Visitech 2000, Visitech Systems, NC)

2.3.5 Murine unilateral hindlimb ischaemia model

Surgery was performed to create unilateral hindlimb ischaemia in mice as previously described (Couffinhal *et al.*, 1998). Mice were anesthetized with ketamine (80 mg/kg IP) and xylazine (16 mg/kg IP). An incision was made over the left inguinal region. The left superficial and deep femoral arteries were carefully dissected and ligated (Figure 2). A section of superficial femoral artery was then also excised. Consequently, blood flow to the ischaemic lower limb was completely dependent on collateral vessels arising from the internal iliac artery and its branches. A sham procedure was performed on the right side. Incisions were closed with surgical staples.

At the end of the experiment, mice were sacrificed by anaesthetic overdose and cervical dislocation. Adductor muscles from both thighs were removed, rinsed in PBS to remove excess blood, placed on dry ice and immediately stored at -80°C until used. Samples were then homogenized and used for western blotting, measurements of NOS activity and ADMA measurements as described above. For all experiments, ischaemic muscle was compared to non-ischaemic muscle on the contralateral side from the same mouse.

Figure 2. Unilateral hindlimb ischaemia in mice was induced by deep and superficial femoral artery ligation and excision under general anaesthesia.

2.4 Human Study

2.4.1 Drugs

ADMA was obtained from Paragon Biochemical (Salt Lake City, US), checked for purity by HPLC and proven to be endotoxin free (Biowhittaker, Belgium). ADMA was dissolved in sterile physiological saline, stored at –80°C, and sterile filtered (0.22μm Millex-GP, Millipore, France) immediately before use. Placebo (sterile physiological saline) was also stored at –80°C.

2.4.2 Haemodynamic measurements

All studies were done with subjects lying supine and with continuous electrocardiographic monitoring. Heart rate was measured continuously by electrocardiography. Systolic and diastolic blood pressures (SBP, DBP) were measured every five minutes using a semi-automated sphygmomanometer placed over the right arm. Mean arterial pressure was calculated as DBP + one-third of the pulse pressure. Continuous cardiac output (CO) measurements were made non-invasively using a bioimpedance cardiograph device (Physio Flow, Manatec Biomedical, France). This method uses changes in transthoracic impedance during cardiac ejection to calculate stroke volume (SV), has the advantage of being able to make beat by beat measurements, is not affected by baseline impedance values (related to differences in chest wall morphology, electrode position and/or skin thickness), and has been validated against other reference methods of CO determination (Charloux *et al.*, 2000). Systemic vascular resistance (SVR; dynes.s.cm^{-5}) was calculated according to the standard formula: SVR = 80(MAP-CVP)/CO.

2.5 Statistics

Data are expressed as means ± SEM. Analyses of haemodynamic data were tested using repeated-measures ANOVA. Comparisons between two groups were evaluated using the Student's t test. $P<0.05$ was considered statistically significant.

Chapter Three: Retinoic Acid regulates NO synthesis and ADMA metabolism

3.1 Introduction

Derivatives of vitamin A, and in particular all-*trans*-retinoic acid (atRA), have profound effects on cardiovascular development and angiogenesis. Chronic vitamin A deficiency and the embryonic deletion of retinoid receptors are associated with malformations of the heart and great vessels (Lammer *et al.*, 1985; Colbert *et al.*, 1997). In the adult cardiovascular system, atRA inhibits neointima formation following vascular injury or bypass grafting (Wiegman *et al.*, 2000; DeRose *et al.*, 1999; Leville *et al.*, 2000; Lee *et al.*, 2000) and vitamin A may retard the development of atherosclerosis (Shaish *et al.*, 1995). Although previous work has focused on atRA effects on vascular smooth muscle cells (VSMC) (Neuville *et al.*, 1999), there are grounds to suspect that atRA may also act via the endothelium. Endothelial cells are exposed to the highest concentrations of circulating atRA, express retinoid receptors and metabolise atRA to a significant degree when compared to other cell types (Lansink *et al.*, 1999). atRA also modulates endothelial cell growth, differentiation and morphology (Braunhut *et al.*, 1991; Lechardeur *et al.*, 1995). Recent data suggests that vitamin A may regulate VSMC function via an unknown endothelial factor (Wang *et al.*, 1997).

NO has been implicated in the regulation of development and cellular differentiation (Peunova *et al.*, 1995). NO effects on endothelial cell migration, angiogenesis, and vascular remodelling are similar to those of atRA (Ziche *et al.*, 1994; Rudic *et al.*, 1998). NO has also been implicated in the atRA-induced differentiation of neuronal cells (Ghigo *et al.*, 1998). We therefore speculated that atRA might also regulate NO production by endothelial cells. With respect to possible mechanisms involved, we focused on the role of asymmetric dimethylarginine (ADMA), an endogenous

inhibitor of NO synthase (NOS) (Vallance *et al.*, 1992). ADMA has been described as an inhibitor of angiogenesis (Jang *et al.*, 2000) and is metabolised by the enzyme DDAH. It has been suggested that changes in DDAH activity may indirectly regulate NO production (MacAllister *et al.*, 1996; Ito *et al.*, 1999). We hypothesised that the ADMA/DDAH pathway might participate in the regulation of NO synthesis by atRA.

3.2 Protocols

Endothelial cell monolayers were grown in 6-well tissue culture plates (approximately 10^6 cells/well at the time of treatment). Murine endothelioma cells (sEnd.1), PAEC and SGHEC-7 cells were treated with atRA. atRA (Sigma) stock solutions were made up in DMSO and added to the medium to achieve a final concentration of 1μM for sEnd.1. Control cells were treated with DMSO only. Final DMSO concentrations in culture medium did not exceed 0.1% (v/v). In some experiments the DDAH inhibitor, S-2-amino-4 (3-methylguanidino) butanoic acid (4124W) (MacAllister *et al.*, 1996), ADMA or symmetric dimethylarginine (SDMA) were added to the culture medium (final concentration 1mM). In other experiments, cells were exposed to actinomycin D (1.0μg/ml) to block transcription.

Total RNA was isolated from confluent cell monolayers grown and subjected to electrophoresis in denaturing agarose gels followed by northern blotting, using probes for DDAH1, DDAH2, eNOS, nNOS and iNOS. Signals were visualised using a phosphorimager and hybridisation with β–actin cDNA was used to control for the amount of RNA isolated and loaded. sEnd.1 cells grown to confluence in 75 cm^2 flasks were subjected to western blotting using a monoclonal antibody (mAb) specific for DDAH2. NO production by sEnd.1 cells was determined by measuring nitrite

accumulation in cell culture medium (Griess reaction). Methylarginine concentrations in conditioned medium were determined by HPLC as previously described (Vallance *et al.*, 1992).

3.3 Results

3.3.1 Effect of atRA on NO synthesis and NOS expression

Nitrite production by sEnd.1 cells increased from 9.9±0.6 to 13.7±0.8 nmol/10^6 cells over 24 hours following stimulation by atRA (1μM) (n=9; P<0.005) (Figure 1). No significant increase in nitrite generation was seen at 4, 8 (data not shown) or 12 hours. Nitrite was undetectable in media from stimulated and control cells treated with the NOS inhibitor ADMA (n=4), confirming that nitrite arose from NOS activity. The inactive enantiomer of ADMA, SDMA, did not affect nitrite accumulation (data not shown).

To determine whether the time-dependent changes in nitrite production might be associated with changes in NOS expression following atRA treatment, we studied NOS mRNA expression in sEnd.1 cells by northern blotting. eNOS mRNA levels decreased by 5.8% and 20.3% at 12 and 24 hours respectively (Figure 2). Expression of nNOS or iNOS mRNA was not detectable at any time point.

3.3.2 Effect of atRA on DDAH expression

Low levels of DDAH1 mRNA were detectable in sEnd.1 cells and no significant changes in expression were seen between control and atRA treated cells over the time

course studied. In contrast, DDAH2 mRNA levels in sEnd.1 cells increased in a time-dependent manner following atRA treatment (by 90% and 73% compared to controls at 12 and 24h respectively) (Figure 2). Western blotting confirmed that DDAH2 protein expression was also increased by atRA treatment of sEnd.1 cells (Figure 2). A similar effect was also seen in the human endothelial cell line SGHEC-7 (data not shown). To test whether atRA also induced DDAH2 expression in primary arterial endothelial cells, we used PAECs. In primary PAECs, atRA increased DDAH2 mRNA expression by 26.3±7.6% ($P<0.01$; n=6) 24h following treatment. atRA treatment of ECV304 cells increased DDAH2 mRNA expression by 45.1±11.9% after 24h ($P<0.02$; n=3). This effect of atRA was blocked by actinomycin D (Figure 3).

3.3.3 Effect of DDAH regulation on methylarginine production

atRA (10μM) treatment of sEnd.1D2A cells for 48h caused a substantial and significant decrease in the ADMA/SDMA ratio in conditioned medium, characteristic of increased DDAH activity (Control=14.14±1.9 vs atRA-treated=9.63±0.8, $P<0.005$, n=16 from three independent experiments) (Figure 4). The absolute concentration of SDMA increased, suggesting increased protein turnover in atRA treated cells.

3.3.4 Effect of DDAH regulation on NO synthesis

To study whether or not the induction of DDAH2 might contribute to the increase in nitrite production following atRA treatment, we used the DDAH inhibitor 4124W which we have previously characterised and shown to alter vascular reactivity in

organ bath studies (MacAllister *et al.*, 1996). In this experiment, 4124W reduced nitrite release from control cells by a small but statistically insignificant amount (n=4). In contrast, 4124W reduced the atRA-stimulated increase in nitrite production by 53.5±9.2% (n=4; $P<0.02$) (Figure 5).

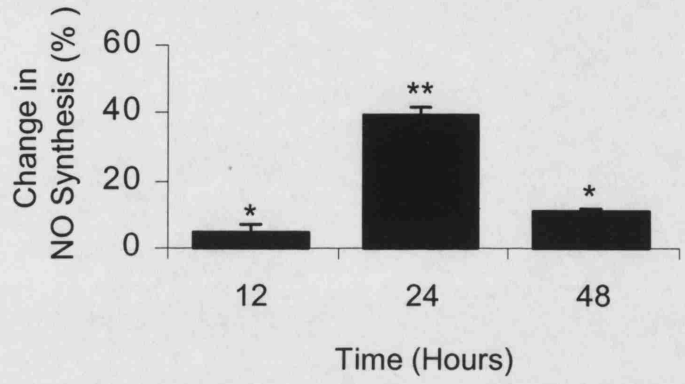

Figure 1. Nitrite production by sEnd.1 cells stimulated by atRA (1μM) for 12, 24 and 48 hours. Results are expressed as the % change compared to control cells at the same time point and are presented as mean±SEM. * $P<0.001$ compared to control (n=5 refers to five experiments performed on two separate days). ** $P<0.001$ compared to control (n=9 refers to nine independent experiments).

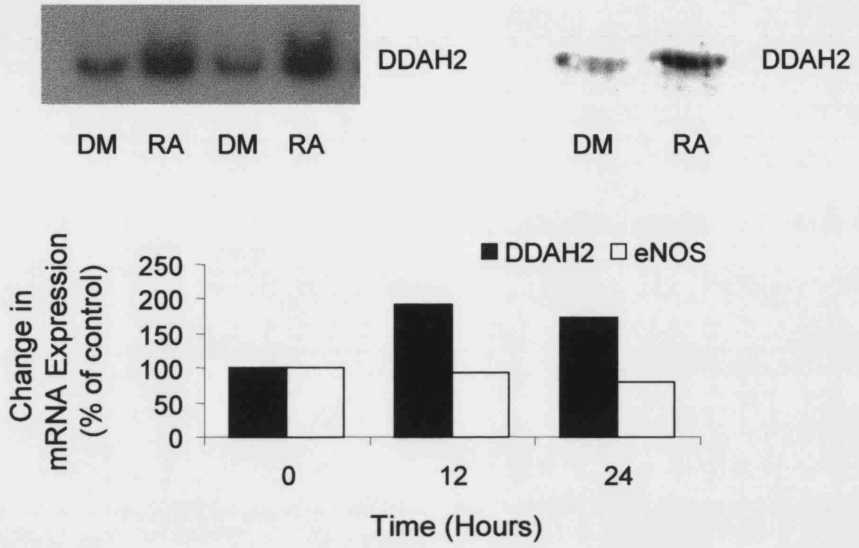

Figure 2. Top Left, Northern analysis of DDAH2 mRNA expression by sEnd.1 cells following DMSO (DM) or RA (1μM) treatment for 24 hours. Top Right, Western analysis of DDAH2 protein expression by sEnd.1 cells following stimulation by atRA (1μM) for 24 hours. Bottom, Expression of DDAH2 (solid bars) and eNOS (open bars) mRNA by sEnd.1 cells following stimulation by atRA (1μM) for 12 and 24 hours. Results are expressed as the % change in mRNA levels compared to control cells at the same time point and have been corrected for differences in actin expression. Values represent means of two separate northern blots using samples from independent experiments.

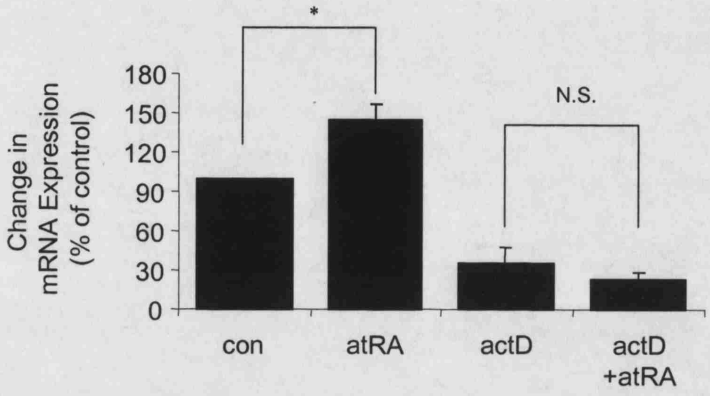

Figure 3. Northern analysis of DDAH2 mRNA expression by ECV304 following stimulation by atRA (1μM) for 24 hours in the presence or absence of actinomycin D. Results are expressed as the % change in mRNA levels compared to control cells and have been corrected for differences in actin expression. *P<0.02 (n=3).

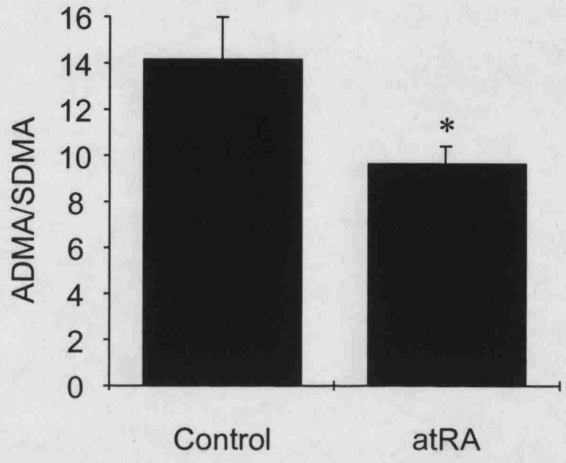

Figure 4. ADMA/SDMA production by atRA treated sEnd.1 cells. sEnd.1D2A cells were treated with 10μM atRA for 48h and methylarginine concentrations in the conditioned medium determined by HPLC. Data are presented as the ADMA/SDMA ratio in culture medium. *P<0.005 (n=16 from three independent experiments).

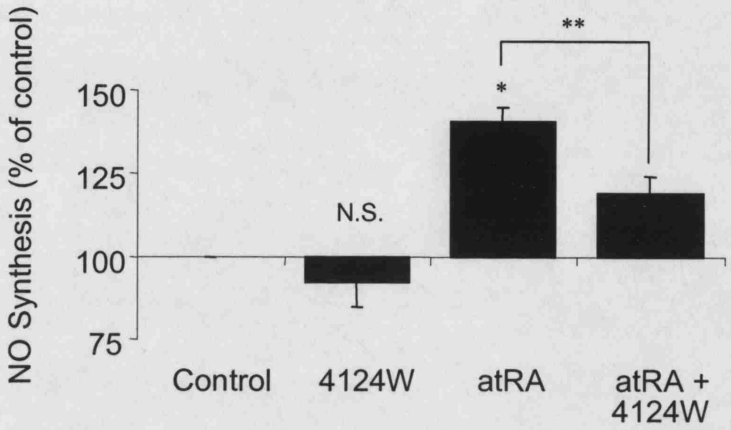

Figure 5. Nitrite production by resting and atRA-stimulated sEnd.1 cells in the presence or absence of the DDAH inhibitor 4124W at 24 hours. Results are expressed as the % change compared to control cells treated with neither atRA nor 4124W. *P<0.005 compared to control. **P<0.02 compared to RA treated group. Results are presented as mean±SEM (n=4).

3.4 Discussion

Retinoic acid is important in the embryonic development of the cardiovascular system (Colbert *et al.*, 1997) and can influence angiogenesis (Lansink *et al.*, 1998). It has recently been shown that atRA treatment may be beneficial in models of cardiovascular disease (Wiegman *et al.*, 2000; DeRose *et al.*, 1999; Leville *et al.*, 2000; Lee *et al.*, 2000; Miano *et al.*, 1998) although the mechanisms involved are not understood. The present study demonstrates that atRA increases nitrite production by endothelial cells in a time dependent manner and suggests that the upregulation of the enzyme DDAH2 in endothelial cells contributes to this effect. These findings describe a novel vascular effect of atRA and clearly identify this retinoid as the first known transcriptional modulator of DDAH2.

A maximum increase in nitrite production by sEnd.1 cells (39.7±2.2%) was seen 24 hours after stimulation with atRA ($n=9$; $P<0.001$). atRA produces many of its effects by regulation of transcription and the time course over which atRA increased nitrite production was consistent with a genomic effect. We therefore studied the effects of atRA on NOS transcription. Neither nNOS nor iNOS mRNA was detectable in sEnd.1 cells and eNOS mRNA expression did not increase following atRA treatment. Indeed a decrease in NOS expression following atRA treatment has previously been described in other cell types (Norford *et al.*, 1998; Datta *et al.*, 1999).

An alternative mechanism for the action of atRA might be the induction of another enzyme that indirectly influences the activity of expressed NOS. Our studies focused on those enzymes which regulate ADMA metabolism, namely the DDAH enzymes. ADMA is an endogenous and competitive inhibitor of NOS which is derived from the degradation of intracellular proteins. The metabolism of ADMA to citrulline by

DDAH may play an important role in the regulation of NO synthesis (MacAllister *et al.*, 1996; Ito *et al.*, 1999).

DDAH1 was expressed at low levels in endothelial cells and was not altered by atRA. However, a second isoform of DDAH (DDAH2) is highly expressed in cardiovascular tissues and has a tissue distribution with similarities to that of eNOS (Leiper *et al.*, 1999). DDAH2 expression was induced by atRA with the maximum increase seen 12 hours following atRA stimulation. A similar effect was seen in primary PAECs, and the human cell lines SGHEC-7 and ECV304. Studies with actinomycin D confirmed that the effect was likely to be due to transcriptional regulation rather than the alteration of mRNA stability. This is consistent with the observation that in ECV304 cells transfected with a reporter construct driven by the human DDAH2 promoter, atRA treatment increased promoter activity significantly (see reprint at the back). These findings are also consistent with the observation that a mouse cDNA sequence (7u), which we identified as DDAH2, is indirectly upregulated in growth arrested melanoma cells treated with atRA (Spanjaard *et al.*, 1997). Establishing the precise molecular mechanism of this indirect atRA effect would be of interest as manipulation of ADMA levels is seen as a potential therapeutic approach in certain disease states (Leiper *et al.*, 1999).

ADMA is a substrate for DDAH while SDMA, also produced during protein turnover, is not. The ADMA/SDMA ratio therefore reflects DDAH activity and was decreased in cell culture medium following atRA treatment, consistent with an increase in DDAH activity. In order to establish whether the increased NO synthesis might be related to the induction of DDAH2 and metabolism of ADMA, we used the DDAH inhibitor 4124W. In the present study, 4124W did not significantly affect nitrite

production by unstimulated sEnd.1 cells, indicating that basal NO synthesis may not be regulated by DDAH activity in these cells. In contrast, 4124W significantly decreased nitrite production in atRA stimulated cells, reducing the increase in nitrite production attributable to atRA stimulation by 53.5±9.2%. At the concentration used, 4124W has previously been shown to inhibit purified DDAH activity by about 40 to 50% (MacAllister *et al.*, 1996) and it is possible that complete inhibition of DDAH activity would reduce NO synthesis to baseline levels in stimulated endothelial cells. Alternatively, it is possible that other mechanisms may also contribute to the induction of NO synthesis by atRA. It is not clear whether the induction of DDAH facilitates NO generation by ensuring that ADMA levels do not rise in response to increased protein turnover, or whether it causes a local fall in ADMA levels at the sites of NO generation. Either way, the experiments using the DDAH inhibitor show that induction of DDAH may be essential for atRA induced NO generation in cell culture. Further studies will be required to determine whether DDAH induction drives or simply facilitates increased NO production.

The regulation of cardiovascular development and function by atRA is of potential physiological and therapeutic importance. Endothelial cells are exposed *in vivo* to relatively high concentrations (approximately 10^{-6} M) of circulating retinol (Wahrendorf *et al.*, 1986). The actions of atRA within the cell are regulated by cytoplasmic RA binding proteins and RA receptors, all of which are expressed and subject to transcriptional regulation in human endothelial cells (Kooistra *et al.*, 1995). Wang *et al.* have demonstrated that retinol can influence vascular smooth muscle function through an endothelium-dependent mechanism (Wang *et al.*, 1997). The results of this study demonstrate that (1) atRA increases NO synthesis by endothelial cells despite no increase in eNOS mRNA expression, (2) atRA induces the expression

of DDAH2 by endothelial cells and reduces the ADMA/SDMA ratio, and (3) the inhibition of DDAH significantly reduces NO synthesis in atRA stimulated cells only. We infer from this that the regulation of DDAH2 expression by atRA may in turn regulate NO synthesis by endothelial cells. Raised ADMA levels are associated with endothelial dysfunction and atherosclerosis (Boger *et al.*, 1998; Miyazaki *et al.*, 1999) and intracellular concentrations of ADMA are increased within endothelial cells that repopulate denuded areas following experimental balloon injury (Azuma *et al.*, 1995). Induction of DDAH2 may lower ADMA levels and restore NO production in these and other conditions.

Acknowledgments

I am grateful to Dr J Kabir for preparation of the PAECs.

Chapter Four: DDAH overexpression in a transgenic mouse model regulates NO synthesis and lowers blood pressure

4.1 Introduction

One approach to the study of gene and protein function in intact physiological systems is the use of genetically engineered animals. Gain and loss of function strategies in animals, by the transgenic overexpression and targeted ablation of candidate genes respectively, have been used to understand the functional importance of genes and proteins *in vivo*. The transgenic mouse is of particular value because its genome is well characterized and can be manipulated with relative ease. The effects of altered gene expression in mice can be studied over prolonged periods of time. Limitations of such an approach include the observation that transgenic proteins may be overexpressed to levels far in excess of those seen in normal physiology.

Genetically modified mice have been to use to study NO physiology. Experiments involving hypertensive mice lacking the eNOS gene and hypotensive mice overexpressing the eNOS gene demonstrate a pivotal role of NO in blood pressure regulation. eNOS knockout mice are hypertensive with mean arterial blood pressures 20 to 30mmHg greater than wild type values and have a significant bradycardia (Huang *et al.*, 1995; Shesely *et al.*, 1996). Conversely, systolic, diastolic and mean arterial pressures in conscious transgenic mice overexpressing bovine eNOS under the control of an endothelium specific promoter are approximately 20mmHg lower than those in control animals (Ohashi *et al.*, 1998).

It has been suggested that DDAH may regulate NO synthesis indirectly. The pharmacological inhibition of DDAH impairs NO synthesis and constricts aortic rings in organ baths (MacAllister *et al.*, 1994). The previous chapter described the regulation of DDAH2 and NO formation by atRA *in vitro*. Experiments described in this chapter tested the hypothesis that changes in ADMA metabolism by DDAH *in*

vivo might regulate cardiovascular function. Using a novel transgenic mouse model, the effects of DDAH overexpression *in vivo* on plasma ADMA levels, tissue NOS activity, and blood pressure were studied.

4.2 Protocols

Transgenic mice overexpressing human DDAH1 (under the control of a human β-actin promoter) were made by the Transgenic Research Facility at Stanford University. DDAH1 mice were made on a C57BL/6J genetic background, and crossed with C57BL/6J mice so that all transgenic mice used in subsequent experiments were heterozygous for the transgene. Transgenic mice were compared to age, weight and sex matched wildtype C57BL/6J controls obtained from parental stocks used to make the transgenic mice. Blood was obtained from anesthetized mice by direct cardiac puncture prior to euthanasia and subjected to ADMA measurements as described above. Tissue homogenates were subjected to sonication on ice and then used for western blotting, measurements of NOS activity and DDAH activity as described previously. Urine was collected for measurement of nitrite and nitrate and urinary creatinine.

Systolic blood pressure was measured non-invasively in conscious mice by tail plethysmography (Visitech 2000, Visitech Systems, Apex, NC). Six male transgenic mice were compared to six sex-matched, age-matched, and weight-matched wildtype controls.

4.3 Results

Transgenic mice did not differ from control littermates in general appearance or behaviour. Transgenic animals developed normally and were fertile. Autopsies on transgenic mice were performed by an experienced veterinarian (Dr D Bouley, Stanford University Department of Comparative Medicine). No gross lesions or abnormalities were observed. Histopathological examination revealed no abnormalities of the heart, arteries or veins. Routine blood tests in the transgenic animals were within normal limits.

4.3.1 Expression and activity of human DDAH1

Western blotting with a murine monoclonal anti-human DDAH1 Ab which does not cross react with endogenous mouse DDAH I confirmed expression of human DDAH1 (35kDa) in homogenates of transgenic tissues (Figure 1). DDAH activity was significantly increased from 0.336±0.107 to 0.916±0.224 μmol citrulline/μg protein/min ($P<0.05$; n=8) in normal and transgenic skeletal muscle respectively (Figure 2A).

4.3.2 Plasma ADMA, SDMA and Arginine Measurements

Plasma ADMA levels were significantly reduced in transgenic mice (0.726±0.085 vs 1.507±0.193μM, $P<0.001$, n=5). Plasma SDMA levels were not significantly different between the two groups (0.922±0.042 vs 0.954±0.103μM, $P>0.5$, n=5) (Figure 2B). As DDAH metabolizes ADMA but not SDMA, this finding is consistent with an

increase in DDAH activity in transgenic mice. Furthermore, plasma arginine levels did not differ between the two groups (27.68±1.65 vs 31.44±3.4μM, P>0.3, n = 5).

4.3.3 Tissue NOS Activity and Expression

No differences in NOS expression between transgenic and normal tissues were apparent on western blotting.

NOS activity was significantly increased in transgenic skeletal muscle homogenates compared to normal (0.195±0.018 vs 0.099±0.017 counts/μg protein/min, P<0.005, n=6) (Figure 3). NOS activity was also increased in transgenic liver homogenates (data not shown). An increase in NOS activity in heart muscle could not be demonstrated.

4.3.4 Measurement of Urinary Nitrogen Oxides

No significant difference was seen between urinary nitrite and nitrate in transgenic and control animals after correction for creatinine (2.85±0.25 transgenic vs 2.52±0.59 control, P>0.05, n=4)

4.3.5 Blood Pressure

To avoid potential gender differences in NO synthesis, all blood pressure measurements were carried out in male mice. The mean systolic blood pressure of

transgenic mice was 6.8mmHg lower than that of age and weight-matched wildtype controls (105.92±1.82 vs 112.71±1.86mmHg, $P<0.05$, n=6) (Figure 4).

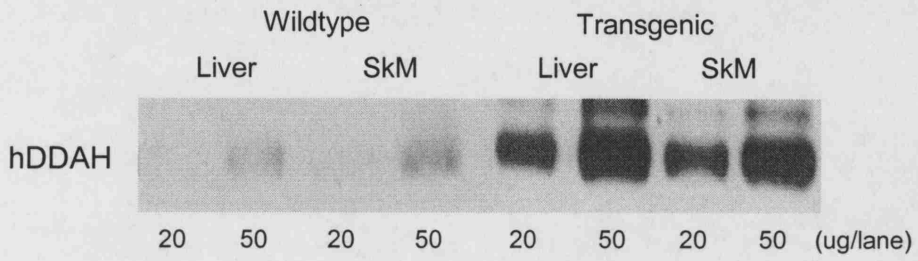

Figure 1. Western blotting, using low (20 μg/lane) and standard amounts (50 μg/lane) of protein, shows increased expression of human DDAH1 in liver and skeletal muscle obtained from transgenic mice compared to normal controls.

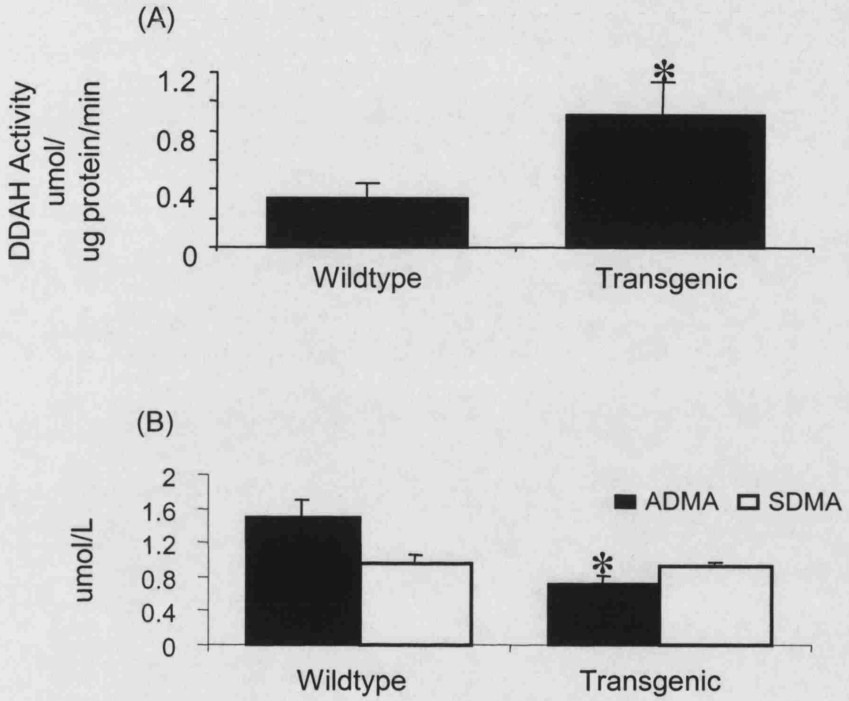

Figure 2. (A) DDAH activity is significantly increased in transgenic skeletal muscle ($P<0.05$, n=8). **(B)** Plasma ADMA, but not plasma SDMA, is significantly reduced in transgenic mice ($P<0.001$, n=5).

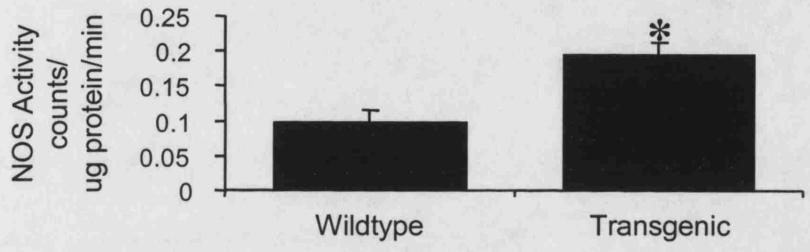

Figure 3. NOS activity is significantly increased in transgenic skeletal muscle as determined by ^{3}H-arginine to ^{3}H-citrulline conversion ($P<0.005$, $n=6$).

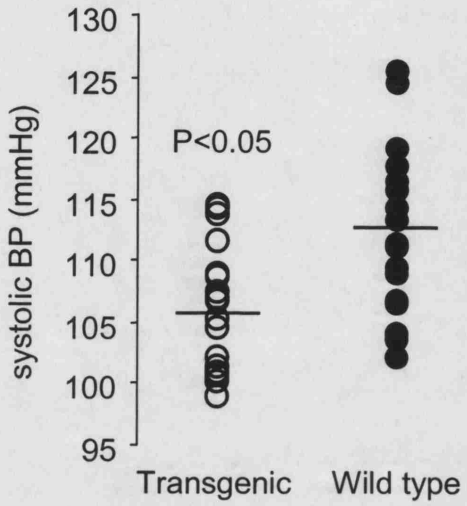

Figure 4. Systolic blood pressure in conscious DDAH overexpression mice is significantly reduced as determined by tail cuff plethysmography ($P<0.05$, $n=6$). Six mice in each group were studied over three days. All eighteen measurements taken over three days are shown in the scatter plot (○ transgenic; ● normal). For calculations and statistical analyses the mean values for each mouse (from three daily measurements) were used.

4.4 Discussion

NO regulates cardiovascular function *in vivo*. Data described in the previous chapter and elsewhere suggests that ADMA metabolism by DDAH may indirectly regulate NO (MacAllister *et al.*, 1996). This study demonstrates that DDAH overexpression *in vivo* reduces circulating ADMA (but not SDMA) and can increase tissue NOS activity; SDMA, unlike ADMA, does not inhibit NOS and is not metabolized by DDAH. These changes are associated with a small but significant fall in systolic blood pressure, roughly half of that seen in mice overexpressing eNOS (Ohashi *et al.*, 1998).

Genetically engineered animal models represent a powerful genetic approach to understanding the functional importance of candidate genes *in vivo*. Systolic, diastolic and mean arterial pressures in conscious transgenic mice overexpressing bovine eNOS under the control of an endothelium specific promoter are approximately 20 mmHg lower than those in control animals (Ohashi *et al.*, 1998). eNOS overexpressing mice also demonstrate reduced vasorelaxation in response to exogenous NO which may be a result of nitrate tolerance following chronic eNOS overexpression, the mechanisms of which are unclear. eNOS overexpression also attenuates congestive heart failure in mice following myocardial infarction (Jones *et al.*, 2004).

The transgenic overexpression approach described here does have some limitations (Hogan *et al.*, 1994). Temporal and spatial patterns of transgene expression, as well as levels of transgene expression, are variable and influenced by the chromosomal location and DNA sequences flanking the insertion site. Random transgene insertion may alter endogenous genes (insertional mutagenesis). Overexpression of the protein

of interest may exceed physiologically relevant levels. Exaggerated physiological effects of transgene overexpression demonstrate only that the gene or protein of interest is capable of an observed effect but alone cannot establish their normal physiological role. Also, adaptive responses to genetic modification may confound interpretation of phenotypes. It should be noted that the phenotype in these experiments is a function of the planned genetic modification and of responses secondary to that perturbation. Many of these problems can be overcome by the generation of independent animal lines and the assessment of dose-response relationships (where the transgene has been expressed to different levels of abundance in separate lines). In order to establish the normal physiological role of the candidate gene, the results of these experiments must be combined with data from future studies.

Several studies have shown an association between elevated ADMA levels and hypertension (Matsuoka *et al.*, 1997; Goonasekera *et al.*, 2000; Fujiwara *et al.*, 2000). Plasma ADMA levels and blood pressure can be regulated by changes in dietary salt intake (Fujiwara *et al.*, 2000). Despite the limitations described above, this study demonstrates for the first time that DDAH overexpression *in vivo* alters circulating ADMA concentrations and produces a measurable effect on systolic blood pressure. DDAH overexpression also appears to increase tissue NO synthesis although this effect could not be demonstrated in all tissue types. Overall, this data supports the view that ADMA metabolism by DDAH may regulate NO synthesis *in vivo*. It also shows that manipulation of the ADMA/DDAH pathway *in vivo* over a long period of time may lead to favourable effects on the cardiovascular system.

In conclusion, we have shown that the increased metabolism of endogenous NOS inhibitors by DDAH overexpression lowers plasma ADMA, increases tissue NOS activity and reduces resting systolic blood pressure. Future models of tissue specific DDAH overexpression perhaps at specified time points may provide additional information. Pharmacological and molecular approaches that increase DDAH expression (and/or activity) may have therapeutic value in cases of hypertension and vascular dysfunction.

Acknowledgements

I am grateful to Dr Y Chen-Tsai (Transgenic Research Facility, Stanford University) for embryo microinjection and making of the transgenic mice. I am also grateful to Dr D Bouley (Stanford University Department of Comparative Medicine) for performing detailed autopsies on the transgenic mice.

Chapter Five: ADMA metabolism by DDAH regulates NO synthesis following hindlimb ischaemia

5.1 Introduction

Acute arterial occlusion due to thrombus, embolus, or trauma results in ischaemia and possible infarction. The severity of ischaemia and the risk of infarction depend to a large extent on the functional capacity of an existing collateral circulation. This expansion of collaterals to salvage a region of ischaemia depends on their number and size as well as their ability to dilate. NO formation plays a key role in the ability of collateral vessels to expand as well as in the structural remodelling of blood vessels due to changes in blood flow (Rudic *et al.*, 1998). Growing evidence suggests that NO also plays a central role in angiogenesis or the formation of new vessels following ischaemia (Ziche *et al.*, 1997; Murohara *et al.*, 1998). NO signalling mediates the regulation of endothelial function and angiogenesis by vascular endothelial growth factor (VEGF; Papapetropoulos *et al.*, 1997; reviewed in Cooke and Losordo, 2002). NO may also play an important role in ischaemic preconditioning (Rakhit *et al.*, 1999). At the same time, NO can be a potent mediator of tissue injury by reacting with superoxide to form peroxynitrite, a potent oxidant. The regulation of NO synthesis during ischaemia is therefore of considerable interest.

Tissue injury leads to an increase in protein turnover. Proteolysis is also essential during the early stages of angiogenesis in order to allow endothelial cells to penetrate ischaemic tissue. It is possible that acute tissue ischaemia might lead to an increase in ADMA formation and thereby threaten NO formation. Data presented in this thesis and elsewhere suggests that ADMA metabolism by DDAH may indirectly regulate NO synthesis and that this pathway may be particularly important when protein turnover is increased (MacAllister *et al.*, 1996; Ito *et al.*, 1999). The following experiments tested the hypothesis that ADMA metabolism by DDAHs may regulate

NO synthesis following acute arterial occlusion and skeletal muscle ischaemia. An established mouse model of unilateral hindlimb ischaemia (Murohara *et al.*, 1998; Couffinhal *et al.*, 1999) was used to look at changes in ADMA formation, protein expression and NO formation at two separate time points.

5.2 Protocols

Surgery was performed to create unilateral hindlimb ischaemia in C57BL/6J mice as previously described (Murohara *et al.*, 1998; Couffinhal *et al.*, 1999). Three and seven days following surgery, mice were sacrificed by anaesthetic overdose and cervical dislocation. Adductor muscles samples were removed, homogenized and used for experiments. Western blotting for DDAH, eNOS, nNOS, iNOS and VEGFR2 was performed. Measurements of NOS activity and ADMA were also carried out. The effect of hindlimb ischaemia in transgenic mice on day 3 was also studied. For all experiments, ischaemic muscle was compared to non-ischaemic muscle on the contralateral side from the same mouse.

5.3 Results

5.3.1 *Wildtype mice*

A murine model of hindlimb ischaemia was used to study the effect of ischaemia on DDAH expression and NOS activity. On day 3 following surgery, NOS activity in ischaemic muscle was reduced by over 80% (0.019±0.004 vs 0.098±0.020 counts/μg protein/min, $P<0.001$, $n = 5$) (Figure 1). This was associated with reduced DDAH

expression as determined by western blotting using a monoclonal Ab specific for mouse DDAH (Figure 4).

A 2-fold elevation of ADMA (10.8±1.2 vs 5.2±0.5 nmol/g protein, $P<0.01$, $n = 5$) (Figure 2) and an approximately 4-fold reduction in the L-arginine : ADMA ratio (12.788±4.867 vs 48.960±9.891, $P<0.05$, $n = 3$) (Figure 3) were measured in ischaemic muscle. We examined nNOS expression using a specific monoclonal Ab (Transduction Laboratories, Lexington, KY) which did not cross react with eNOS or iNOS. As described by other groups (Tatchum *et al.*, 2000), two bands were detected around 160kDa in normal skeletal muscle. While the higher band corresponds with a dark band seen with the positive control (rat cerebellum), others have suggested that the lower band represents one of several nNOS splice variants described in skeletal muscle. Expression of both nNOS variants was reduced in the ischaemic limb on day 3 (Figure 4). Neither iNOS nor eNOS were detectable by western blotting in control or ischaemic samples.

On day 7, there was a 7-fold increase in NOS activity in the ischaemic limb compared to the contralateral control limb (0.545±0.069 vs 0.076±0.017 counts/μg protein/min, $P<0.005$, $n=5$) (Figure 1). nNOS expression either returned to baseline (lower band) or remained lower (higher band) than in the control limb (Figure 4). Once again, neither iNOS nor eNOS were detectable. Intriguingly, the increase in NOS activity was temporally related to an increase in DDAH expression in the ischaemic limb by over 300% ($P<0.05$, $n=6$) in association with a fall in tissue ADMA towards baseline (5.93±1.00 nmol/g protein, $n=5$) (Figure 2).

5.3.2 *Transgenic mice*

We hypothesized that elevated ADMA levels, perhaps secondary to reduced DDAH expression, might account at least in part for the reduction in NOS activity seen on day 3. To test this hypothesis, the effect of hindlimb ischaemia in transgenic mice on day 3 was examined. Tissue ADMA levels on day 3 were lower in transgenic muscle compared to wildtype controls (6.7±1.0 vs 10.8±1.2 nmol/g protein, $P<0.05$, n=5) (Figure 5A).

Despite comparable changes in nNOS expression in control and transgenic mice, NOS activity in the ischaemic limb on day 3 was significantly higher in transgenic mice when compared to controls (0.093±0.017 vs 0.019±0.004 counts/μg protein/min, $P<0.02$, n = 5) (Figure 5B).

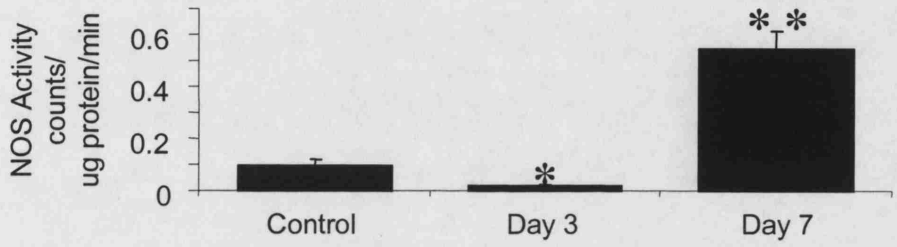

Figure 1. Wildtype skeletal muscle NOS activity following onset of hindlimb ischaemia: NOS activity falls on day 3 (*$P<0.001$, n=5) and then rises by day 7 (**$P<0.005$, n=5).

Figure 2. Skeletal muscle ADMA levels are raised on day 3 (*P<0.01, n=5) and then return to baseline by day 7 (**NS vs control, n=5).

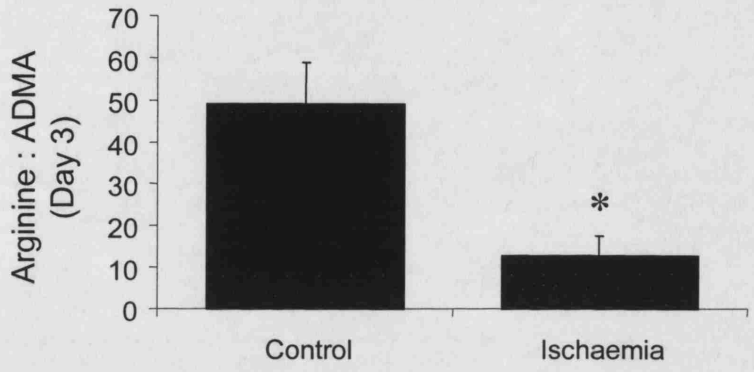

Figure 3. Tissue arginine: ADMA ratio in skeletal muscle falls on day 3 following ischaemia ($P<0.05$, n=3).

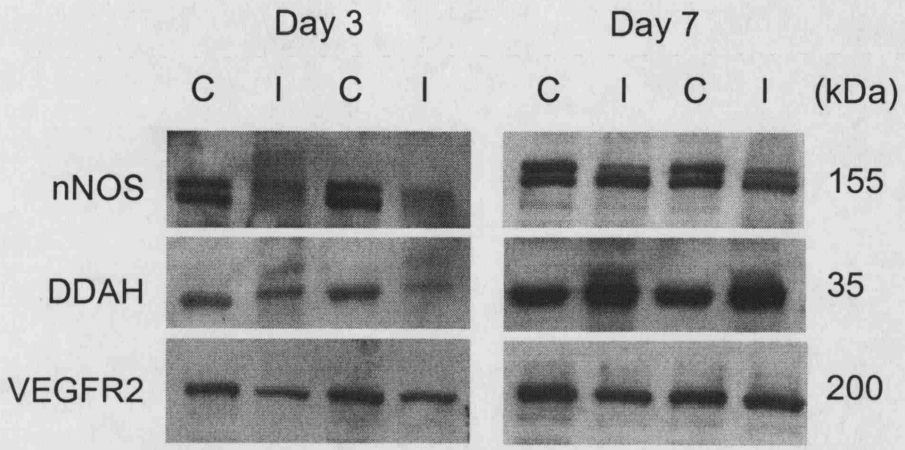

Figure 4. Western blots showing nNOS, DDAH I and VEGFR2 expression in skeletal muscles obtained from control (C) and ischaemic (I) hindlimbs on days 3 and 7 following ischaemia (50 μg protein/lane). nNOS in control skeletal muscle blots as two bands thought to be splice variants. nNOS expression is reduced on day 3 and returns to baseline by day 7. DDAH I expression falls on day 3 but is raised above baseline on day 7. Expression of VEGFR2 which signals via NO is shown for comparison.

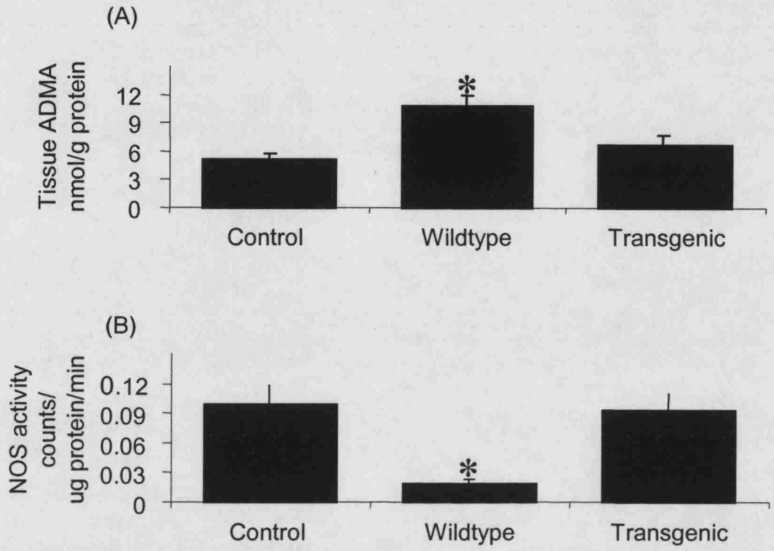

Figure 5. (A) ADMA levels in transgenic muscle on day 3 following ischaemia are lower than in wildtype mice ($P<0.05$, n=5). **(B)** NOS activity of transgenic muscle is increased on day 3 following ischaemia compared to wildtype mice ($P<0.02$, n=5)

5.4 Discussion

Previous data suggests that DDAH may regulate NO synthesis indirectly, both *in vitro* and *in vivo*. Following arterial occlusion and tissue ischaemia, NO mediates tissue injury but also determines the functional capacity of collateral vessels, angiogenesis, and the vascular remodelling of collateral vessels. The regulation of NO formation in response to ischaemia is therefore of considerable interest and has important therapeutic implications. In a murine model of unilateral hindlimb ischaemia, this study shows that ischaemia leads to an early increase in tissue ADMA levels. This is associated with reduced endogenous DDAH1 expression and reduced NOS activity in skeletal muscle. On day 7, however, the upregulation of endogenous DDAH1 is associated with a reduction in tissue ADMA levels and the recovery of NOS activity. The early increase in ADMA levels was significantly blunted when hindlimb ischaemia was induced in transgenic mice overexpressing DDAH1. NOS activity was also preserved in these animals on day 3. As in previous studies, nNOS was identified as the predominant NOS isoform in skeletal muscle. Our data suggests that endogenous DDAH plays a role in the regulation of NO synthesis by nNOS following hindlimb ischaemia.

The role of NO in the response to ischaemia is controversial. Reduced NOS activity increases infarct size following myocardial and cerebral infarction in animals (Sumeray *et al.*, 2000; Kidd *et al.*, 2000). The role of NO in angiogenesis is controversial. Angiogenesis is a complex multistep process that involves endothelial cell migration, proliferation, and differentiation into vascular tubes. Differentiating smooth muscle cells or pericytes then form a surrounding vessel wall and function to stabilise the vascular tubes as well as modulate blood flow. An NO dependent

increase in blood flow may act as an early stimulus for angiogenesis. Conflicting data suggests that NO has both positive and negative effects on the migration of cultured endothelial cells (Ziche *et al.*, 1994; Lau and Ma, 1996). Pipili-Synetos and colleagues (1993) reported that NO donors inhibit angiogenesis in the chick chorioallantoic membrane. Others however have shown that NO regulates tube formation *in vitro* (Ziche *et al.*, 1997). The potent effects of vascular endothelial growth factor (VEGF) on endothelial cell function and angiogenesis via endothelial cell surface receptors (VEGFR-1 and VEGFR-2) are mediated by NO and blocked by NOS inhibitors (Papapetropoulos *et al.*, 1997). Using the model of unilateral hindlimb ischaemia described in this study, Murohara and colleagues have demonstrated that angiogenesis is significantly impaired in mice lacking eNOS; this abnormality was not improved by VEGF administration, consistent with an NO effect downstream to VEGF signalling (Murohara *et al.*, 1998). Coronary collateral growth following repetitive coronary artery occlusion in dogs is dependent on VEGF and NO (Matsunaga *et al.*, 2000). Conversely, eNOS overexpression in mice enhances angiogenesis following hindlimb ischaemia (Amano *et al.*, 2003). Recently, NO has also been shown to suppress the formation of angiostatin, an endogenous antagonist of angiogenesis (Matsunaga *et al.*, 2002). All these factors are likely to contribute to the control of angiogenesis by NO.

Ischaemia leads to tissue injury and changes in protein turnover. Proteolysis of the extracellular matrix is a key early step during angiogenesis (Cai *et al.*, 2000). The activation of matrix metalloproteinases during ischaemia promotes angiogenesis in a rodent hindlimb model (Silvestere *et al.*, 2002). Raised levels of plasma ADMA have been described in patients following poor tissue perfusion during hemorrhagic shock and stroke (Aneman *et al.*, 1994; Yoo *et al.*, 2001). More recently, the accumulation

of ADMA following ischaemia of the rabbit urethra has been described in association with decreased NOS activity and impaired neurogenic relaxation (Masuda *et al.*, 2001). In a rat model of skeletal muscle ischaemia, Jin and colleagues describe the presence of an unidentified NOS inhibitor in plasma obtained from ischaemic hindlimbs (Jin *et al.*, 1995). Concentrations of ADMA in skeletal muscle from hypercholesterolaemic mice are raised when compared to normal muscle; this may account for the impairment of angiogenesis in mice with hypercholesterolaemia (Duan *et al.*, 2000). ADMA inhibits angiogenesis in a disc model of angiogenesis (Jang *et al.*, 2000). This study demonstrates for the first time that ADMA levels increase in skeletal muscle following arterial occlusion and hindlimb ischaemia. Moreover ADMA levels are dynamically regulated by changes in DDAH expression.

The mouse model of unilateral hindlimb ischaemia used in this study is well established (Murohara *et al.*, 1998; Couffinhal *et al.*, 1999). Femoral artery resection has been shown to immediately reduce blood flow to approximately 40%. This effect persists on day 3 and is then followed by a restoration of flow to over 80% by day 14 (Murohara *et al.*, 1998). The temporal expression of VEGF receptor expression over this time corresponds to the recovery of capillary density (Couffinhal *et al.*, 1999). This study looked at ADMA formation, protein expression and NOS activity on days 3 and 7 following unilateral femoral artery occlusion and resection. ADMA levels in tissue significantly increased soon after acute arterial occlusion. This was associated with reduced NO formation. At the later time point (day 7), the upregulation of DDAH1 was associated with a fall in ADMA levels and an increase in NOS activity above baseline. Changes in VEGFR2 (and thereby capillary density; Couffinhal *et al.*, 1999) described in this study correspond with changes described in the literature. In order to explore the significance of these observations, femoral artery ligation was

performed in transgenic mice overexpressing DDAH and changes on day 3 studied. The data suggests that early changes in tissue ADMA levels and NOS activity following ischaemia can be modulated by DDAH overexpression *in vivo*. This supports the hypothesis that DDAH upregulation following ischaemia *in vivo* may at the very least serve to preserve if not facilitate NOS activity.

Data presented earlier in this thesis shows that atRA regulates ADMA metabolism by DDAHs and NO formation by endothelial cells. atRA has also been implicated in the regulation of angiogenesis (Lansink *et al.*, 1998). ADMA inhibits angiogenesis *in vitro* (Jang *et al.*, 2000). DDAH overexpression modulates VEGF expression and cell phenotype *in vitro* (Smith *et al.*, 2003). In a resting dog model of myocardial blood flow, Laussmann and colleagues (2003) have recently shown that DDAH1 expression is markedly increased in low-flow areas of the myocardium and that this is associated with reduced ADMA levels; out of all proteins upregulated in low-flow regions, DDAH1 displayed the maximum relative increase. DDAH overexpression also increases tumour angiogenesis *in vivo* (Kostourou *et al.*, 2002). Our finding that the upregulation of endogenous DDAH modifies ADMA formation and NO synthesis following ischaemia *in vivo* supports the view that DDAH may also regulate angiogenesis following ischaemia. The effect of DDAH overexpression on ischaemia induced angiogenesis *in vivo* at later time points is the subject of future study and studies in a DDAH knockout model are likely to be provide valuable additional data.

Our findings may also have implications for the role of the ADMA/DDAH pathway during functional ischaemia of healthy skeletal muscle. Skeletal muscle force production and the regulation of blood flow, both at rest and during exercise, are dependent on NO (Stamler *et al.*, 2001). Functional ischaemia has been described in

nNOS deficient skeletal muscle in children with Duchenne muscular dystrophy (Sander *et al.*, 2000). Abnormalities of the ADMA/DDAH pathway may also exist in these and similar patients.

In conclusion, the data demonstrates that following hindlimb ischaemia *in vivo*, NOS activity falls and then rises with reciprocal changes in tissue ADMA levels. Tissue ADMA levels are inversely related to DDAH expression. Finally, we show that DDAH overexpression in a transgenic model reduces ADMA accumulation and increases NOS activity on day 3 following ischaemia compared to control C57BL/6J mice. The regulation of tissue ADMA and NO formation by DDAH following arterial occlusion may determine the extent of tissue recovery following the onset of ischaemia and may regulate angiogenesis. DDAH activity can be reduced by oxidative stress, nitrosylation, and hyperglycaemia (Ito *et al.*, 1999; Leiper *et al.*, 2002; Lin *et al.*, 2002). Abnormalities in the ADMA/DDAH pathway may account for reduced NO formation, poor tissue recovery and impaired angiogenesis following ischaemia in certain patient groups. Conversely, pharmacological and molecular approaches that increase DDAH expression or activity may have therapeutic value following acute arterial occlusion such as in myocardial infarction or stroke.

Chapter Six: Cardiovascular effects of ADMA in humans

6.1 Introduction

Raised plasma concentrations of ADMA have been described in association with a range of human cardiovascular diseases. The finding that plasma ADMA levels are elevated in chronic renal failure led to speculation that ADMA may in part be responsible for increased cardiovascular risk and hypertension in these patients (Vallance *et al.*, 1992). Subsequent studies have shown associations between raised ADMA levels and cardiovascular risk factors, endothelial dysfunction, hypertension, and atherosclerosis (Boger *et al.*, 1998; Stuhlinger *et al.*, 2001; Matsuoka *et al.*, 1997; Miyazaki *et al.*, 1999). ADMA levels are also significantly raised in patients with chronic heart failure (Usui *et al.*, 1998), rats with acute heart failure induced by coronary artery ligation (Feng *et al.*, 1998), and dogs with heart failure induced by rapid pacing (Ohnishi *et al.*, 2002). Despite the expanding literature, the significance of these associations is not clear and the systemic cardiovascular effects of ADMA in humans are not known.

The aim of the present study was to test the hypothesis that administration of low dose ADMA to healthy volunteers would produce cardiovascular changes similar to those seen in diseases associated with ADMA accumulation. Data presented in this thesis and elsewhere has shown that ADMA metabolism by DDAHs may regulate NO synthesis both *in vitro* and in animals *in vivo*. The extent of ADMA metabolism by DDAHs in humans however is not known. We also set out to test the hypothesis that ADMA is extensively metabolised in humans.

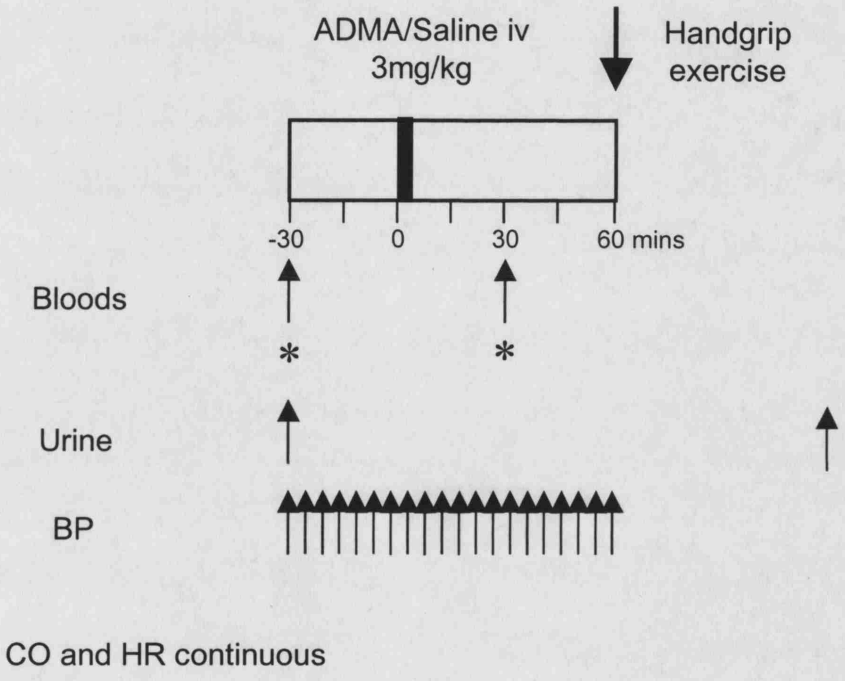

Figure 1. Experimental protocol for human ADMA study.

6.2 Protocol

The following protocol was approved by the University College London Research Ethics Committee. An information sheet was given to volunteers explaining the nature, purpose, and potential risks of this study. Twelve healthy, normotensive men (aged 23 to 42 y.o.) gave informed and signed consent to participate in the study. All studies were performed in a temperature-controlled laboratory (24-26°C).

The study design was randomised, double-blind and placebo-controlled (Figure 1). Volunteers were randomly allocated to receive an intravenous injection of either ADMA (3mg/kg upto a maximum of 250mg; Paragon Biochemical) or placebo (sterile physiological saline). Volunteers were asked to avoid seafood in their diet for 24 hours prior to and during the study in order to minimise dietary DMA intake. 18GA intravenous cannulae were inserted into deep antecubital veins in both arms. Baseline blood samples were collected from the left arm prior. Systolic and diastolic blood pressures (SBP, DBP) were measured every five minutes using a semi-automated sphygmomanometer. Heart rate and cardiac output (CO) were measured non-invasively and continuously. Systemic vascular resistance (SVR; dynes.s.cm^{-5}) was calculated according to the standard formula: SVR = 80(MAP-CVP)/CO. After a 30min phase of baseline haemodynamic measurements in the supine position, subjects received an injection of ADMA or placebo over 30secs followed by a 10ml saline flush via the cannula in the right arm. Haemodynamic measurements were continued for 60mins following injection. At the end of this period, subjects performed a fixed handgrip exercise protocol for a minute using the right (dominant) hand (generating a pressure of 200mmHg 40 times over a minute). Urine samples were collected at the start of the study and two hours following injection. Blood samples were taken at

baseline and 30min. All blood samples were centrifuged and separated immediately. All samples were stored at –80°C until further analysis. Dimethylamine levels in the urine (μM) were determined by the method of Beal and Bryan (1978). Urinary creatinine levels (mg/dL) were measured using a method modified from that of Slot and developed by Sigma Diagnostics. ADMA measurements were made by HPLC.

6.3 Results

No significant differences in baseline weight, heart rate, cardiac output, mean blood pressure, and systemic vascular resistance were found between the two study groups. Of all parameters studied heart rate was the first to change (Figure 2). Intravenous injection of ADMA reduced heart rate by a maximum of 9.2±1.4% from 58.9±2.0 beats per min (after 5mins; $P<0.001$), and reduced cardiac output by a maximum of 14.8±1.2% from 4.4±0.3 L/min (after 10 mins; $P<0.001$). ADMA also increased mean blood pressure by a maximum of 6.0±1.2% from 88.6±3.4mmHg (after 10mins; $P<0.005$), and increased SVR by a maximum of 23.7±2.1% from 1639.0±91.6 dynes.s.cm^{-5} (after 10mins; $P<0.001$) (Figure3). Changes in heart rate and cardiac output returned to baseline during the 60mins following injection. Other parameters approached but did not reach baseline values by the end of the study. No significant changes were seen in those subjects receiving placebo. Handgrip exercise increased cardiac output in control subjects by a maximum of 96.8±23.3%. In contrast, those subjects given ADMA increased their cardiac output by 35.3±10.6% during exercise, representing a significant reduction in the cardiac response to exercise ($P<0.05$) (Figure 4). Blood pressure was recorded at the beginning and end of exercise and did

not change. Heart rate increased in both groups and there was no statistical difference between the groups. No analysis of SVR was undertaken because of the lack of BP measurements during exercise.

In those subjects that received ADMA, the plasma ADMA concentration increased to 2.6±0.3μM at 30min following the injection ($P<0.001$ compared to baseline). Symmetric dimethylarginine (SDMA) levels did not change significantly (data not shown).

Urinary dimethylamine (μM):Cr (mg/dL) ratio was unaltered following placebo but increased from 1.26±0.32 to 2.73±0.59 following ADMA injection ($P<0.05$) (Figure 5). Assuming a normal urinary Cr excretion in men of 1.1 to 2.8g/24h, normal baseline urinary dimethylamine excretion was estimated at 260±30μmol/24h.

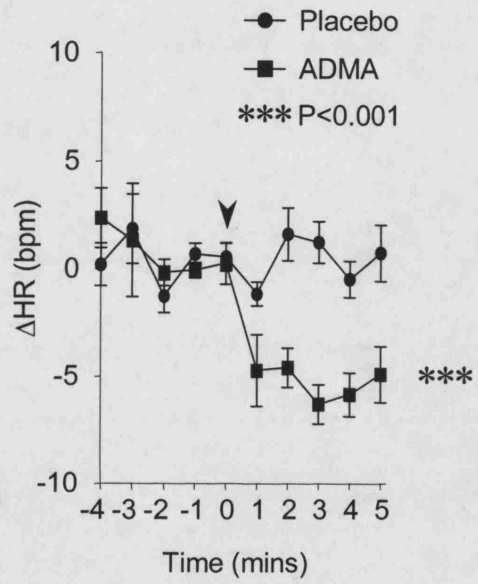

Figure 2. Absolute changes in heart rate from baseline 5min prior to and 5min following intravenous ADMA (3mg/kg) or placebo injection at rest (n=6).

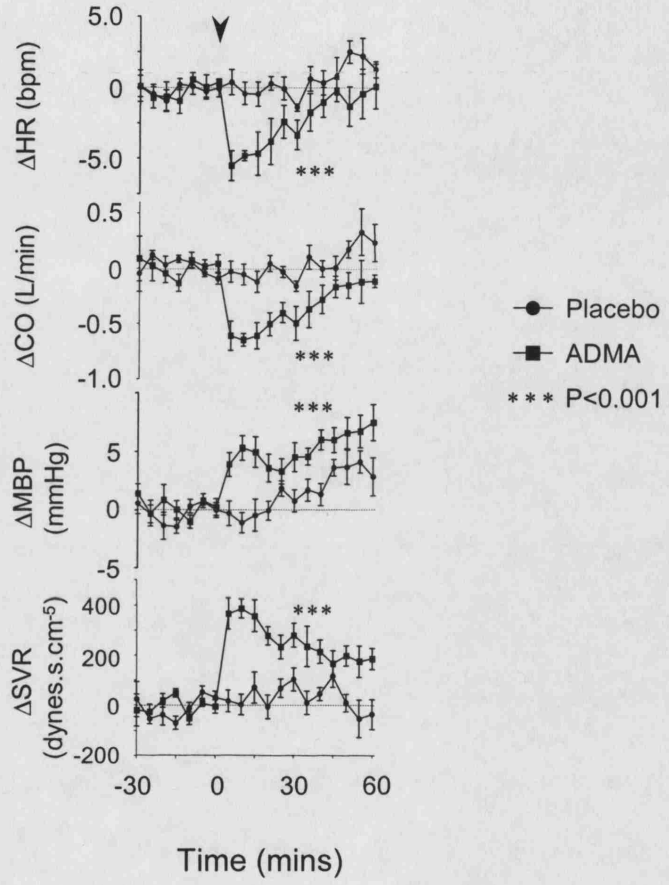

Figure 3. Absolute changes in heart rate (HR), cardiac output (CO), mean blood pressure (MBP) and systemic vascular resistance (SVR) from baseline 30min prior to and 60min following intravenous ADMA (3mg/kg) or placebo injection at rest (n=6) (time of injection is marked by arrow).

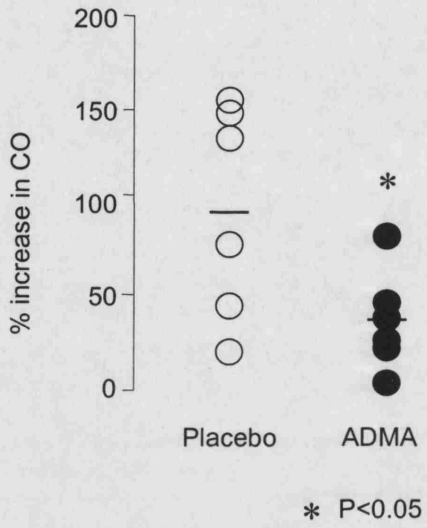

Figure 4. Maximum change in cardiac output (% increase) during one minute of handgrip exercise 60min following intravenous ADMA (3mg/kg) or placebo injection (n=6).

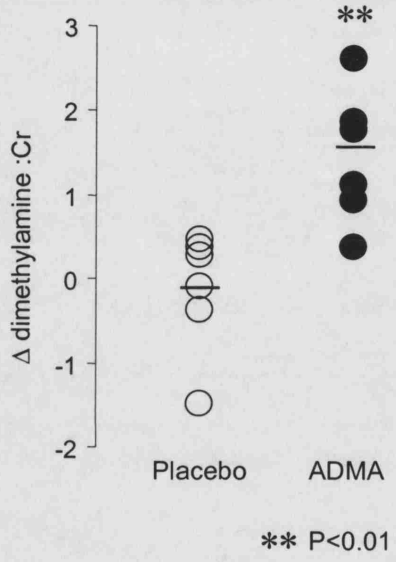

Figure 5. Effect of ADMA or placebo on urinary dimethylamine (μM):Cr(mg/dL) ratio 2hrs following injection (n=6).

6.4 Discussion

This study demonstrates that a systemic increase in the endogenous NOS inhibitor ADMA produces adverse cardiovascular effects in humans. In a randomised, double-blind, placebo-controlled study in healthy volunteers, an intravenous injection of ADMA significantly reduced heart rate and cardiac output, and increased blood pressure and systemic vascular resistance. Furthermore, subjects receiving ADMA showed an impaired cardiac output response to upper limb exercise. Injection of ADMA also increased urinary dimethylamine excretion demonstrating that the ADMA/DDAH pathway is active in humans. Together these results support a causal role relationship between raised ADMA levels and cardiovascular dysfunction and suggest that the metabolism of ADMA by DDAH may be an important regulatory mechanism in the human cardiovascular system.

ADMA is a naturally occurring methylarginine that inhibits all three isoforms of NOS (Vallance *et al.*, 1992). ADMA has been shown to inhibit eNOS *in vitro*, in animals and in the human forearm arterial bed (Vallance *et al.*, 1992). However, the systemic effects of ADMA have not been studied in humans previously. We used a bolus dose of ADMA in order to assess the speed of onset of response and to determine its duration in the face of ongoing ADMA metabolism. The results of this randomised, double-blind, controlled study show clearly that ADMA produces major haemodynamic changes at rest similar, but not identical to those previously reported for L-NMMA (Petros *et al.*, 1991; Haynes *et al.*, 1993). ADMA and L-NMMA are structurally similar and have similar effects on isolated eNOS (Vallance *et al.*, 1992). Nonetheless, differences between ADMA and L-NMMA effects have been reported (Cardounel *et al.*, 2000; Tsikas *et al.*, 2000) and it is important to define the systemic

effects of ADMA in humans *in vivo*. ADMA produced a large increase in systemic vascular resistance in association with a fall in cardiac output, and as a result the overall rise in blood pressure was relatively modest. It is not known whether the change in cardiac output was secondary to the change in blood pressure or whether it represents a direct effect of NOS inhibition on cardiac function (Sarkar *et al.*, 2001). The effect was most marked when cardiac output increased in response to exercise; indeed ADMA infusion markedly blunted this physiological response.

The literature suggests that NO may directly regulate cardiac output as well as blood pressure. Systemic infusions of the pharmacological NOS inhibitors, L-NMMA and L-NAME, increase blood pressure, reduce heart rate and reduce cardiac output in animals, healthy human volunteers as well as in patients with septic shock (Petros *et al.*, 1991; Haynes *et al.*, 1993). eNOS knockout mice are hypertensive, with mean arterial blood pressures 20 to 30 mmHg greater than wild type values, and develop a significant bradycardia (Huang *et al.*, 1995; Shesely *et al.*, 1996). In studies using isolated cardiomyocytes or isolated heart preparations, low physiological (submicromolar) NO concentrations produce positive inotropic and chronotropic effects. At slightly higher (but still physiological) doses, NO may enhance myocyte relaxation and thus diastolic relaxation. By contrast, at higher submillimolar concentrations exogenous NO has pronounced negative inotropic effects (Sarkar *et al.*, 2001). Systemic haemodynamic reflexes complicate the *in vivo* assessment of cardiac contractility. Nevertheless, the intracoronary application of NO donors produces direct positive inotropic effects *in vivo* (Preckel *et al.*, 1997) while intracoronary L-NMMA produces a small reduction in cardiac output *in vivo* independent of changes in cardiac loading (Cotton *et al.*, 2001). In contrast to the above findings, contractile responses to a beta-agonist (isoproterenol) are significantly

increased in eNOS knockout mice and may reflect compensatory changes in the eNOS knockout heart (Gyurko *et al.*, 2000).

It is also interesting to note the most rapid change seen in response to ADMA was a change in heart rate. A significant fall in heart rate was seen almost immediately, and well before blood pressure had changed. This observation is consistent with results from animal studies (Hogan *et al.*, 1999) and suggests an important role for endogenous NO generated from either eNOS or nNOS in the control of heart rate in humans. The findings would not be consistent with the idea that cardiac changes were secondary to changes in blood pressure.

Increased plasma ADMA concentrations occur in a wide range of disease states or risk factors in which cardiovascular events are increased (Boger *et al.*, 1998; Stuhlinger *et al.*, 2001; Matsuoka *et al.*, 1997; Miyazaki *et al.*, 1999), and in some situations there is a clear relationship between the level of ADMA and morbidity or mortality (Zoccali *et al.*, 2001; Zoccali *et al.*, 2002). There is a strong biological rationale for assuming that these relationships are likely to be causal since inhibition of NO enhances atherogenesis and cardiovascular disease in animal models (Naruse *et al.*, 1994; Cayatte *et al.*, 1994). However plasma ADMA levels seldom rise above the low micromolar range and it is not clear that this would be sufficient to affect vascular function, particularly as concentrations of the NOS substrate, arginine, are much higher. Our results show that at a time when circulating concentrations of ADMA are in the low micromolar range, substantial cardiovascular effects are evident and persistent. It is probable that plasma ADMA concentrations were higher immediately following the injection and prior to equilibration within the body compartments. However, since ADMA is generated within endothelial cells, intracellular

concentrations of this compound are also higher than the 'overspill' concentrations found in plasma (Azuma *et al.*, 1995; MacAllister *et al.*, 1996). What the present study demonstrates is that the plasma ADMA concentrations that have been measured in cardiovascular diseases can be associated with prolonged and major cardiovascular effects even in the presence of normal circulating levels of arginine. In future studies it would be interesting to measure ADMA levels at different time points to assess peak levels and the half life of ADMA.

Plasma concentrations of ADMA are increased in patients with renal failure (Vallance *et al.*, 1992), and it is assumed that failed urinary clearance is a major mechanism underlying accumulation. However, ADMA also accumulates in many other diseases in which renal function is normal, and it is ADMA that rises rather than the biologically inactive SDMA (Boger *et al.*, 1998). These observations have led to interest in the role of DDAH as a mechanism to control ADMA levels *in vivo*. DDAH metabolises ADMA (but not SDMA) to dimethylamine and citrulline (Ogawa *et al.*, 1987; Ogawa *et al.*, 1989). Animal studies have indicated that the extensive metabolism of asymmetric methylarginines, but not symmetric methylarginines, takes place *in vivo* but also that significant differences between species may exist (McDermott, 1976; Ogawa *et al.*, 1987; Schwartz *et al.*, 1997). Despite evidence that ADMA metabolism by DDAHs may be the major route for elimination of ADMA and a major determinant of ADMA levels, there have been no studies of *in vivo* DDAH activity in humans to date.

The present study shows a substantial increase in urinary dimethylamine excretion after administration of ADMA and is the first demonstration of DDAH activity in

humans *in vivo*. Our estimate of daily dimethylamine formation (260μmols/24h) is consistent with other studies (Beal and Bryan, 1978; Zhang *et al.*, 1994; Zhang *et al.*, 1995). The bulk of urinary dimethylamine arises from an endogenous source (Zeisel *et al.*, 1985), suggesting that the rate of ADMA metabolism is in the order of 130-260μmols/24h compared to a renal excretion rate of about 60μmols/24h (Vallance *et al.*, 1992). Consistent with these estimates, the average protein turnover is about 300g/24h and the average ADMA content is about 1-2μmol/g protein (Kakimoto *et al.*, 1975), giving an estimated total body production of 300μmols ADMA in 24hrs. Thus, a complete failure of DDAH activity (combined with a loss of renal excretion) might lead to a daily increase in plasma ADMA concentrations by about 5μM (300umol/60L). Oxidative stress, inflammation and nitrosylation have all been shown to reduce DDAH activity (Ito *et al.*, 1999; Stuhlinger *et al.*, 2001; Leiper *et al.*, 2002). It has also been shown that the local inhibition of DDAH in the vessel wall increases ADMA accumulation and inhibits endothelium-dependent relaxation (MacAllister *et al.*, 1996). The current finding of substantial ADMA metabolism to dimethylamine in humans provides evidence that DDAH is active *in vivo* and supports the suggestion that reduced DDAH activity due to oxidative stress or inflammation (Ito *et al.*, 1999; Stuhlinger *et al.*, 2001; Leiper *et al.*, 2002) could raise circulating ADMA levels during disease.

ADMA is formed continuously and actively metabolised by two isoforms of DDAH that are widely expressed in vascular and non-vascular cells (Leiper *et al.*, 1999). This study demonstrates significant cardiovascular effects of exogenous ADMA and shows that these persist at a time when plasma concentrations may be considered rather low. Although we have only studied the acute effects of ADMA, it seems reasonable to assume that chronic exposure to raised ADMA is likely to produce even greater

effects (Naruse *et al.*, 1994; Cayatte *et al.*, 1994). ADMA levels are elevated in patients with heart failure (Usui *et al.*, 1998). In a recent analysis of a large group of patients with end stage renal failure by Zoccali and colleagues, plasma ADMA concentrations emerged as second only to age as a predictor of overall mortality and cardiovascular risk (Zoccali *et al.*, 2001). A further study in the same patient group demonstrated an inverse relationship between ADMA levels and left ventricular (LV) ejection fraction; in a multivariate analysis which included LV end diastolic volume and heart rate, ADMA was shown to be an independent and strong predictor of LV ejection fraction. The results presented here suggest that ADMA may have a direct role in the pathophysiology of certain types of cardiac dysfunction. The effects of ADMA on resting and exercise-stimulated cardiac output described here suggest a possible causal role for ADMA in the pathophysiology of heart failure as well as a mechanism for reduced exercise tolerance in other conditions. The profound effects on heart rate suggest that it would be interesting to determine whether ADMA levels correlate with heart rate changes or altered heart rate regulation in those conditions in which ADMA levels are increased. Finally the demonstration of significant ADMA metabolism by DDAH in humans *in vivo* suggests that abnormalities in the ADMA/DDAH pathway may be harmful and that DDAH represents a target for future therapeutic strategies.

Chapter Seven: Discussion

NO is a key mediator of the cardiovascular system and controls vascular tone, blood pressure, heart rate and cardiac contractility. Abnormalities in NOS regulation are thought to contribute to the pathogenesis of many cardiovascular disorders. The regulation of NO synthesis is complex and there is growing interest in the role of endogenous arginine analogues. ADMA inhibits all three isoforms of NOS and is formed during protein breakdown within cells. An unknown proportion of ADMA escapes into extracellular compartment and can be detected in plasma and urine (Vallance *et al.*, 1992). The discovery that plasma ADMA concentrations are increased in renal failure patients led to speculation that abnormal levels of ADMA might account for cardiovascular abnormalities in man; this study also demonstrated that a local infusion of ADMA into the brachial artery led to a reduction in forearm blood flow (Vallance *et al.*, 1992). Recent data supports the view that plasma ADMA concentrations may be a strong predictor of overall mortality and cardiovascular risk in this patient group (Zoccali *et al.*, 2001). In the absence of renal impairment, associations between ADMA concentrations and coronary risk, endothelial dysfunction, and chronic heart failure have been shown (Valkonen *et al.*, 2001; Usui *et al.*, 1998).

Animal studies demonstrate that ADMA is extensively metabolised *in vivo* and that significant species differences exist (McDermott, 1976; Ogawa *et al.*, 1987; Schwartz *et al.*, 1997). Two isoforms of the enzyme DDAH metabolise ADMA *in vitro* and of these DDAH2 is widely expressed in the cardiovascular system (Leiper *et al.*, 1999). The pharmacological inhibition of DDAH increases ADMA accumulation in cell culture medium, impairs NO synthesis by cultured cells, and causes the endothelium dependent contraction of rat aortic rings in organ baths studies (MacAllister *et al.*, 1996). These findings suggest that the metabolism of ADMA to citrulline by DDAHs

may indirectly regulate NO synthesis. The experiments described in this thesis support the hypothesis that the ADMA/DDAH pathway might regulate NOS activity *in vitro* and *in vivo* and demonstrate the possible role of this pathway both in animals and man, as well as during ischaemia, *in vivo*.

The first series of experiments *in vitro* demonstrate an increase in DDAH2 expression and nitrite formation by endothelial cells treated with atRA. The data strongly suggests that DDAH2 indirectly regulate NO synthesis *in vitro*. atRA increased nitrite production by endothelial cells by approximately 40% after 24 hours, consistent with a genomic effect. No effect on NOS expression was seen while DDAH2 expression almost doubled 12 hours following atRA stimulation. That the induction of DDAH2 might be essential for atRA induced NO generation was confirmed with the help of DDAH specific inhibitor. These findings are consistent with the observation that a mouse cDNA sequence (7u), which we have now identified as DDAH2, was indirectly upregulated in growth arrested melanoma cells treated with atRA (Spanjaard *et al.*, 1997). These findings describe a novel vascular effect of atRA and clearly identify this retinoid as the first known transcriptional modulator of DDAH2. They also demonstrate for the first time that the transcriptional regulation of DDAHs may regulate NO synthesis.

Endothelial cells are exposed *in vivo* to relatively high concentrations (approximately 10^{-6}M) of circulating vitamin A (Wahrendorf *et al.*, 1986). The actions of atRA within the cell are regulated by cytoplasmic RA binding proteins and RA receptors, all of which are expressed and subject to transcriptional regulation in human endothelial cells (Kooistra *et al.*, 1995). atRA reduces neointima formation following vascular injury and regulates angiogenesis (Ziche *et al.*, 1994; Rudic *et al.*, 1998). Some of

these actions may be endothelium dependent (Wang *et al.*, 1997). atRA can regulate neuronal differentiation via NO (Ghigo *et al.*, 1998). The observation that atRA regulates NO formation by endothelial cells suggests that atRA effects on the cardiovascular system may also be NO mediated. The involvement of ADMA metabolism by DDAH2 supports the hypothesis that DDAH regulates NO synthesis and also illustrates the following point. ADMA formation during protein breakdown may pose a constant threat to NO formation. This threat may be further magnified during differentiation and angiogenesis when protein turnover is increased; the upregulation of DDAH by atRA in such situations may facilitate NO signalling..

DDAH inhibitor studies using cells and vascular rings (MacAllister *et al.*, 1996) and the atRA data described above strongly indicate that ADMA metabolism by DDAH may regulate NO synthesis *in vitro*. Several studies have demonstrated the metabolism of asymmetric methylarginines including ADMA in animals (McDermott, 1976; Ogawa *et al.*, 1987). The significance of ADMA metabolism by DDAH *in vivo* however is not known.

The second series of experiments described in this thesis uses a genetically engineered animal model to test the functional effects of ADMA metabolism by DDAH *in vivo*. Transgenic mice overexpressing DDAH1 were characterised to test the haemodynamic effects of increased ADMA metabolism *in vivo*. Chronic DDAH1 overexpression *in vivo* increased tissue DDAH activity, reduced plasma ADMA but not SDMA concentrations, and increased tissue NOS activity. Furthermore, DDAH overexpression significantly lowered systolic blood pressure in conscious mice as measured using a non-invasive approach. This data supports the view that ADMA metabolism by DDAHs may regulate NO and that manipulation of this pathway *in*

vivo may have important haemodynamic effects. These findings may have important implications for the control of blood pressure in man.

ADMA metabolism by DDAHs may also regulate NOS function in pathological states. The atRA data suggests that ADMA metabolism may be important in conditions associated with increased protein turnover. One such condition associated with increased protein turnover is tissue injury following ischaemia. Raised plasma ADMA concentrations have recently been described in patients following cerebral ischaemia (Yoo *et al.*, 2001). The accumulation of ADMA following ischaemia of the rabbit urethra has been described in association with decreased NOS activity and impaired neurogenic relaxation (Masuda *et al.*, 2001). During ischaemia, NO determines the functional capacity of collateral vessels, regulates vascular remodelling of blood vessels in response to changes in blood flow, and promotes angiogenesis. The role of NO in ischaemia-induced angiogenesis has been demonstrated using a mouse model of unilateral hindlimb ischaemia (Murohara *et al.*, 1998). The same model was used in the third series of experiments presented here to test the hypothesis that the ADMA/DDAH pathway might regulate of NOS following acute arterial occlusion and ischaemia *in vivo*.

ADMA formation, protein expression and NOS activity were studied on days 3 and 7 following unilateral femoral artery occlusion and resection. Skeletal muscle tissue ADMA concentrations increased soon after acute arterial occlusion. This was associated with reduced NO formation. On day 7 however, the upregulation of DDAH1 coincided with a reduction in tissue ADMA levels and an increase in NOS activity. Although it is not possible to conclude that the upregulation of DDAH was essential for the observed increase in NOS activity, the data strongly supports the

view that a reduction in tissue at the very least preserves NO synthesis. When the same experiments were performed in transgenic mice overexpressing DDAH, the ADMA increase was blunted and NOS activity preserved even on day 3.

Once again, this data supports the hypothesis that ADMA metabolism by DDAH regulates NOS *in vivo* and also suggests that this pathway may be particularly important following arterial occlusion and ischaemia. Growing evidence suggests that NO mediates the action of VEGF and other angiogenic factors during angiogenesis (Papapetropoulos *et al.*, 1997; reviewed in Cooke and Losordo, 2002). DDAH may modulate this signalling pathway and DDAH overexpression may have important biological effects. Indeed, enhanced tube formation *in vitro* and tumour angiogenesis *in vivo* following DDAH overexpression have been described recently (Smith *et al.*, 2003; Kostourou *et al.*, 2002). The recent description of DDAH upregulation in low-flow areas of the dog heart is also consistent with these findings (Laussmann *et al.*, 2002).

The above data from a variety of approaches support the hypothesis that ADMA metabolism by DDAHs regulates NOS. A number of studies have shown an association between raised plasma and tissue ADMA levels and animal models of human disease including vascular injury and heart failure. There is an assumption that these relationships are likely to be causal since inhibition of NO enhances atherogenesis and cardiovascular disease in animal models (Naruse *et al.*, 1994; Cayatte *et al.*, 1994). Associations between raised plasma ADMA levels (in the low micromolar range) and patients with cardiovascular disease have been described. These associations are strongest in patients with renal impairment but also appear in those patients with normal renal function. Forearm blood flow falls significantly

following local infusions of ADMA into the brachial artery in healthy human subjects (Vallance *et al.*, 1992). These data suggest that ADMA may be a mediator, rather than simply a marker, of abnormal cardiovascular function. The systemic effects of an increase in ADMA in man however are not known. Furthermore, despite growing evidence that ADMA metabolism takes place in animals and may be functionally important, there are no studies looking at the metabolism of ADMA by DDAH in humans *in vivo*.

The final study in this thesis demonstrates that a systemic increase in ADMA produces adverse cardiovascular effects in humans. In a randomised, double-blind, placebo-controlled study in healthy volunteers, an intravenous injection of ADMA significantly reduced heart rate and cardiac output (by 9.2% and 14.8% respectively), and increased blood pressure and systemic vascular resistance (by 6.0% and 23.7% respectively). At a time when circulating concentrations of ADMA were in the low micromolar range, substantial cardiovascular effects were evident and persistent. These findings are consistent with cardiovascular abnormalities described in association with raised ADMA levels in patients. Furthermore, subjects receiving ADMA showed an impaired cardiac output response to upper limb exercise. Finally, injection of ADMA also increased urinary dimethylamine excretion demonstrating for the first time that the ADMA/DDAH pathway is active in humans *in vivo*. Using two separate approaches, we estimated that the body produces approximately 300μmols of ADMA per day, of which approximately 250μmols is metabolised to dimethylamine by DDAH. Together these results support a causal role relationship between raised ADMA levels and cardiovascular dysfunction and suggest that the metabolism of ADMA by DDAH may be an important regulatory mechanism in the human cardiovascular system. In the complete absence of ADMA metabolism, we predict

that tissue and plasma concentrations of ADMA would rise to harmful levels. The effects of ADMA on resting and exercise-stimulated cardiac output described here suggest a possible causal role for ADMA in the pathophysiology of heart failure as well as a mechanism for reduced exercise tolerance in human disease.

In summary, the studies described in this thesis support the hypothesis that ADMA and its metabolism by DDAH regulate NO synthesis *in vitro* and *in vivo*. We have shown atRA upregulates DDAH2 and that this increases NO synthesis. The genetic overexpression of DDAH in a transgenic mouse model also increases NO synthesis and lowers blood pressure *in vivo*. Data using a mouse model of unilateral hindlimb ischaemia shows that ischaemia increases ADMA formation *in vivo* and also that the subsequent upregulation of DDAH coincides with a normalisation of tissue ADMA levels and restoration of NO synthesis. This supports our hypothesis that the regulation of ADMA metabolism may have a particular significance in situations associated with increased protein turnover. Finally, a randomised, double-blind, placebo-controlled study has shown that an acute systemic increase in ADMA produces adverse cardiovascular effects in humans, both at rest and during exercise, and that these persist at a time when plasma concentrations may be considered rather low. These results support a causal role relationship between raised ADMA levels and cardiovascular disorders. Smaller but chronic increases in ADMA are also likely to have adverse effects. The demonstration of significant ADMA metabolism by DDAH in humans *in vivo* suggests that the ADMA/DDAH pathway may be important in the regulation of NO formation in humans, and that findings in animal models may be extrapolated to human subjects.

Abnormalities in the ADMA/DDAH pathway may be harmful and may account for cardiovascular abnormalities in human disease. The data presented here suggests that pharmacological and molecular approaches to increase DDAH expression may have therapeutic value in hypertension and following myocardial or cerebral ischaemia and infarction. Studies looking at the manipulation of ADMA metabolism in healthy volunteers and cardiovascular patients are likely to be of considerable interest and may lead to future cardiovascular therapies.

Chapter Eight: References

Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J* 2001; 357(Pt 3): 593-615

Aletta JM, Cimato TR, Ettinger MJ. Protein methylation: a signal event in post-translational modification. *Trends Biochem Sci* 1998; 23(3): 89-91

Amano K, Matsubara H, Iba O, Okigaki M, Fujiyama S, Imada T, Kojima H, Nozawa Y, Kawashima S, Yokoyama M, Iwasaka T. Enhancement of ischemia-induced angiogenesis by eNOS overexpression. *Hypertension* 2003; 41(1): 156-62

Aneman A, Backman V, Snygg J, von Bothmer C, Fandriks L, Pettersson A. Accumulation of an endogenous inhibitor of nitric oxide synthase during graded hemorrhagic shock. *Circ Shock* 1994; 44(3): 111-4

Arnold WP, Mittal CK, Katsuki S, Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. *Proc Natl Acad Sci U S A* 1977; 74(8): 3203-7

Azuma H, Sato J, Hamasaki H, Sugimoto A, Isotani E, Obayashi S. Accumulation of endogenous inhibitors for nitric oxide synthesis and decreased content of L-arginine in regenerated endothelial cells. *Br J Pharmacol* 1995; 115(6): 1001-4

Beal DD, Bryan GT. Quantitative spectrophotometric determination of human and rat urinary dimethylamine. *Biochem Med* 1978;19:374-82

Blum A, Hathaway L, Mincemoyer R, Schenke WH, Kirby M, Csako G, Waclawiw MA, Panza JA, Cannon RO 3rd. Oral L-arginine in patients with coronary artery disease on medical management. *Circulation* 2000; 101(18): 2160-4

Boger RH, Bode-Boger SM, Brandes RP, Phivthong-ngam L, Bohme M, Nafe R, Mugge A, Frolich JC. Dietary L-arginine reduces the progression of atherosclerosis in cholesterol-fed rabbits: comparison with lovastatin. *Circulation* 1997; 96: 1282-90

Boger RH, Bode-Boger SM, Szuba A, Tsao PS, Chan JR, Tangphao O, Blaschke TF, Cooke JP. Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. *Circulation* 1998; 98(18): 1842-7

Boger RH, Bode-Boger SM, Tsao PS, Lin PS, Chan JR, Cooke JP. An endogenous inhibitor of nitric oxide synthase regulates endothelial adhesiveness for monocytes. *J Am Coll Cardiol* 2000; 36(7): 2287-95

Bogle RG, MacAllister RJ, Whitley GS, Vallance P. Induction of NG-monomethyl-L-arginine uptake: a mechanism for differential inhibition of NO synthases? *Am J Physiol* 1995; 269(3 Pt 1): C750-6

Braunhut SJ, Palomares M. Modulation of endothelial cell shape and growth by retinoids. *Microvasc Res* 1991; 41(1): 47-62

Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 1990; 347(6295): 768-70

Cai W, Vosschulte R, Afsah-Hedjri A, Koltai S, Kocsis E, Scholz D, Kostin S, Schaper W, Schaper J. Altered balance between extracellular proteolysis and antiproteolysis is associated with adaptive coronary arteriogenesis. *J Mol Cell Cardiol* 2000 Jun; 32(6): 997-1011

Cardounel AJ, Xia Y, Zweier JL. Different effects of endogenous nitric oxide synthase inhibitors on nitric oxide and superoxide production (abstract). *Circulation* 2000; 102: II-117

Cayatte AJ, Palacino JJ, Horten K, Cohen RA. Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler Thromb* 1994; 14(5): 753-9

Celermajer DS, Sorensen KE, Bull C, Robinson J, Deanfield JE. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am Coll Cardiol* 1994; 24(6): 1468-74

Channon KM, Qian H, Neplioueva V, Blazing MA, Olmez E, Shetty GA, Youngblood SA, Pawloski J, McMahon T, Stamler JS, George SE. *In vivo* gene transfer of nitric oxide synthase enhances vasomotor function in carotid arteries from normal and cholesterol-Fed rabbits. *Circulation* 1998; 98(18): 1905-11

Charloux A, Lonsdorfer-Wolf E, Richard R, Lampert E, Oswald-Mammosser M, Mettauer B, Geny B, Lonsdorfer J. A new impedance cardiograph device for the non-invasive evaluation of cardiac output at rest and during exercise: comparison with the direct Fick method. *Eur J Appl Physiol* 2000; 82: 313-20

Cimato TR, Tang J, Xu Y, Guarnaccia C, Herschman HR, Pongor S, Aletta JM. Nerve growth factor-mediated increases in protein methylation occur predominantly at type I arginine methylation sites and involve protein arginine methyltransferase 1. *J Neurosci Res* 2002; 67(4): 435-42

Clarkson P, Celermajer DS, Powe AJ, Donald AE, Henry RM, Deanfield JE. Endothelium-dependent dilatation is impaired in young healthy subjects with a family history of premature coronary disease. *Circulation* 1997; 96(10): 3378-83

Colbert MC, Hall DG, Kimball TR, Witt SA, Lorenz JN, Kirby ML, Hewett TE, Klevitsky R, Robbins J. Cardiac compartment-specific overexpression of a modified retinoic acid receptor produces dilated cardiomyopathy and congestive heart failure in transgenic mice. *J Clin Invest* 1997; 100(8): 1958-68

Cooke JP, Dzau VJ. Nitric oxide synthase: Role in the genesis of vascular disease. *Annu Rev Med* 1997; 48: 489-509

Cooke JP, Losordo DW. Nitric oxide and angiogenesis. *Circulation* 2002; 105(18): 2133-5

Cooke JP, Rossitch E, Andon N, Loscalzo J, Dzau VJ. Flow activates an endothelial potassium channel to release an endogenous nitrovasodilator. *J Clin Invest* 1991; 88: 1663

Cooke JP, Singer AH, Tsao P, Zera P, Rowan RA, Billingham ME. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. *J Clin Invest* 1992; 90(3): 1168-72

Cotton JM, Kearney MT, MacCarthy PA, Grocott-Mason RM, McClean DR, Heymes C, Richardson PJ, Shah AM. Effects of nitric oxide synthase inhibition on basal function and the force-frequency relationship in the normal and failing human heart *in vivo*. *Circulation* 2001; 104(19): 2318-23

Couffinhal T, Silver M, Zheng LP, Kearney M , Witzenbichler B , Isner JM. A mouse model of angiogenesis. *Am. J. Pathol.* 1998; 152: 1667-1679

Creager MA, Girerd XJ, Gallagher SJ, Coleman S, Dzau VJ, Cooke JP. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J Clin Invest* 1992; 90: 1248-1253

Cuthbertson DP, in *Human Protein Metabolism* 1964, Vol. II, Munro HN and Allison JB (eds), New York, Academic Press, pp. 373-414

Datta PK, Lianos EA. Retinoic acids inhibit inducible nitric oxide synthase expression in mesangial cells. *Kidney Int* 1999; 56(2): 486-93

De Caterina R, Libby P, Peng HB, Thannickal VJ, Rajavashisth TB, Gimbrone MA Jr, Shin WS, Liao JK. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 1995; 96: 60-8

DeRose JJ Jr, Madigan J, Umana JP, Prystowsky JH, Nowygrod R, Oz MC, Todd GJ. Retinoic acid suppresses intimal hyperplasia and prevents vessel remodeling following arterial injury. *Cardiovasc Surg* 1999; 7(6): 633-9

Dollery CM, McEwan JR, Henney AM. Matrix metalloproteinases and cardiovascular disease. *Circ Res* 1995; 77(5): 863-8

Drexler H, Zeiher AM, Meinzer K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet* 1991; 338: 1546-50

Duan J, Murohara T, Ikeda H, Katoh A, Shintani S, Sasaki K, Kawata H, Yamamoto N, Imaizumi T. Hypercholesterolemia inhibits angiogenesis in response to hindlimb ischemia: nitric oxide-dependent mechanism. *Circulation* 2000 Nov 7; 102(19 Suppl 3): III370-6

Egashira K, Hirooka Y, Kuga T, Mohri M, Takeshita A. Effects of L-arginine supplementation on endothelium-dependent coronary vasodilation in patients with angina pectoris and normal coronary arteriograms. *Circulation* 1996; 94(2): 130-4

Faraci FM, Brian JE Jr, Heistad DD. Response of cerebral blood vessels to an endogenous inhibitor of nitric oxide synthase. *Am J Physiol* 1995; 269(5 Pt 2): H1522-7

Feldman LJ, Mazighi M, Scheuble A, Deux JF, De Benedetti E, Badier-Commander C, Brambilla E, Henin D, Steg PG, Jacob MP. Differential expression of matrix metalloproteinases after stent implantation and balloon angioplasty in the hypercholesterolemic rabbit. *Circulation* 2001; 103(25): 3117-22

Feng Q, Lu X, Fortin AJ, Pettersson A, Hedner T, Kline RL, Arnold JM. Elevation of an endogenous inhibitor of nitric oxide synthesis in experimental congestive heart failure. *Cardiovasc Res* 1998; 37: 667-75

Flam BR, Hartmann PJ, Harrell-Booth M, Solomonson LP, Eichler DC. Caveolar localization of arginine regeneration enzymes, argininosuccinate synthase, and lyase, with endothelial nitric oxide synthase. *Nitric Oxide* 2001; 5(2): 187-97

Fujiwara N, Osanai T, Kamada T, Katoh T, Takahashi K, Okumura K. Study on the relationship between plasma nitrite and nitrate level and salt sensitivity in human hypertension: modulation of nitric oxide synthesis by salt intake. *Circulation* 2000; 101(8): 856-61

Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; 288(5789): 373-6

Ghigo D, Priotto C, Migliorino D, Geromin D, Franchino C, Todde R, Costamagna C, Pescarmona G, Bosia A. Retinoic acid-induced differentiation in a human neuroblastoma cell line is associated with an increase in nitric oxide synthesis. *Cell Physiol* 1998;174(1):99-106

Giugliano D, Marfella R, Verrazzo G, Acampora R, Coppola L, Cozzolino D, D'Onofrio F. The vascular effects of L-Arginine in humans. The role of endogenous insulin. *J Clin Invest* 1997; 99(3): 433-8

Goonasekera CD, Shah V, Rees DD, Dillon MJ. Vascular endothelial cell activation associated with increased plasma asymmetric dimethyl arginine in children and young adults with hypertension: a basis for atheroma? *Blood Press* 2000; 9(1): 16-21

Gyurko R, Kuhlencordt P, Fishman MC, Huang PL. Modulation of mouse cardiac function *in vivo* by eNOS and ANP. *Am J Physiol Heart Circ Physiol* 2000; 278(3): H971-81

Haynes WG, Noon JP, Walker BR, Webb DJ. L-NMMA increases blood pressure in man. *Lancet* 1993; 342(8876): 931-2

Hecker M, Mitchell JA, Harris HJ, Katsura M, Thiemermann C, Vane JR. Endothelial cells metabolize NG-monomethyl-L-arginine to L-citrulline and subsequently to L-arginine. *Biochem Biophys Res Commun* 1990; 167(3): 1037-43

Heinegard D, Tiderstrom G. Determination of serum creatinine by a direct colorimetric method. *Clin Chim Acta* 1973; 43: 305-10

Hibbs JB Jr, Vavrin Z, Taintor RR. L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J Immunol* 1987; 38(2): 550-65

Hingorani AD, Liang CF, Fatibene J, Lyon A, Monteith S, Parsons A, Haydock S, Hopper RV, Stephens NG, O'Shaughnessy KM, Brown MJ. A common variant of the endothelial nitric oxide synthase (Glu298>Asp) is a major risk factor for coronary artery disease in the UK. *Circulation* 1999; 100: 1515-20

Hogan B, Beddington R, Costantini F, Lacy E. *Manipulating the Mouse Embryo: A Laboratory Manual* 1994, Cold Spring Harbor Laboratory Press

Hogan N, Kardos A, Paterson DJ, Casadei B. Effect of exogenous nitric oxide on baroreflex function in humans. *Am J Physiol* 1999; 277(1 Pt 2): H221-7

Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC. Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 1995; 377(6546): 239-42

Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci U S A* 1987; 84(24): 9265-9

Ignarro LJ, Cirino G, Casini A, Napoli C. Nitric oxide as a signaling molecule in the vascular system: an overview. *J Cardiovasc Pharmacol* 1999; 34(6): 879-86

Ito A, Tsao PS, Adimoolam S, Kimoto M, Ogawa T, Cooke JP. Novel mechanism for endothelial dysfunction: dysregulation of dimethylarginine dimethylaminohydrolase. *Circulation* 1999; 99(24): 3092-5

Jang J, Ho H-K, Kwan HH, Adimoolam, S, Fajardo LF, Cooke JP. Angiogenesis is impaired by hypercholesterolemia: Role of asymmetric dimethylarginine *Circulation* 2000; 102: 1414-9

Jin JS, Webb RC, D'Alecy LG. Inhibition of vascular nitric oxide-cGMP pathway by plasma from ischemic hindlimb of rats. *Am J Physiol* 1995; 269(1 Pt 2): H254-61

Jones SP, Greer JJ, Kakkar AK, Ware PD, Turnage RH, Hicks M, van Haperen R, de Crom R, Kawashima S, Yokoyama M, Lefer DJ. Endothelial nitric oxide synthase overexpression attenuates myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol* 2004; 286(1): H276-82

Kakimoto Y, Matsuoka Y, Miyake M, Konishi H. Methylated amino acid residues of proteins of brain and other organs. *J Neurochem* 1975;24(5):893-902

Kidd GA, Dobrucki LW, Brovkovych V, Bohr DF, Malinski T. Nitric oxide deficiency contributes to large cerebral infarct size. *Hypertension* 2000; 35(5): 1111-8

Kooistra T, Lansink M, Arts J, Sitter T, Toet K. Involvement of retinoic acid receptor alpha in the stimulation of tissue-type plasminogen-activator gene expression in human endothelial cells. *Eur J Biochem.* 1995; 232(2): 425-32

Kostourou V, Robinson SP, Cartwright JE, Whitley GS. Dimethylarginine dimethylaminohydrolase I enhances tumour growth and angiogenesis. *Br J Cancer* 2002; 87(6): 673-80

Krege JH, Hodgin JB, Hagaman JR, Smithies O. A noninvasive computerized tail-cuff system for measuring blood pressure in mice. *Hypertension* 1995; 25(5): 1111-5

Kuhlencordt PJ, Gyurko R, Han F, Scherrer-Crosbie M, Aretz TH, Hajjar R, Picard MH, Huang PL. Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E/endothelial nitric oxide synthase double-knockout mice. *Circulation* 2001; 104(4): 448-54

Lammer EJ, Chen DT, Hoar RM, Agnish ND, Benke PJ, Braun JT, Curry CJ, Fernhoff PM, Grix AW Jr, Lott IT. Retinoic acid embryopathy. *N Engl J Med* 1985; 313(14): 837-41

Lansink M, Koolwijk P, van-Hinsbergh V, Kooistra T. Effect of steroid hormones and retinoids on the formation of capillary-like tubular structures of human microvascular endothelial cells in fibrin matrices is related to urokinase expression. *Blood* 1998; 92(3): 927-38

Lansink M, van Bennekum AM, Blaner WS, Kooistra T. Differences in metabolism and isomerization of all-trans-retinoic acid and 9-cis-retinoic acid between human endothelial cells and hepatocytes. *Eur J Biochem* 1999; 247(2): 596-604

Lau YT, Ma WC. Nitric oxide inhibits migration of cultured endothelial cells. *Biochem Biophys Res Commun* 1996; 221(3): 670-4

Laussmann T, Janosi RA, Fingas CD, Schlieper GR, Schlack W, Schrader J, Decking UK. Myocardial proteome analysis reveals reduced NOS inhibition and enhanced glycolytic capacity in areas of low local blood flow. *FASEB J* 2002; 16(6): 628-30

Lechardeur D, Schwartz B, Paulin D, Scherman D. Induction of blood-brain barrier differentiation in a rat brain-derived endothelial cell line. *Exp Cell Res* 1995; 220(1): 161-70

Lee CW, Park SJ, Park SW, Kim JJ, Hong MK, Song JK. All-trans-retinoic acid attenuates neointima formation with acceleration of reendothelialization in balloon-injured rat aorta. *J Korean Med Sci* 2000; 15(1): 31-6

Leiper JM, Santa Maria J, Chubb A, MacAllister RJ, Charles IG, Whitley GS, Vallance P. Identification of two human dimethylarginine dimethyl-aminohydrolases with distinct tissue distributions and homology with microbial arginine deiminases. *Biochem J* 1999; 343(1): 209-214

Leville CD, Dassow MS, Seabrook GR, Jean-Claude JM, Towne JB, Cambria RA. All-Trans-Retinoic Acid Decreases Vein Graft Intimal Hyperplasia and Matrix Metalloproteinase Activity *in vivo*. *J Surg Res* 2000; 90(2): 183-190

Libby P, Schonbeck U. Drilling for oxygen: angiogenesis involves proteolysis of the extracellular matrix. *Circ Res* 2001 Aug 3; 89(3): 195-7

Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. *Circulation* 2001; 104(3): 365-72

Lin KY, Ito A, Asagami T, Tsao PS, Adimoolam S, Kimoto M, Tsuji H, Reaven GM, Cooke JP. Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. *Circulation* 2002; 106(8): 987-92

Lou MF. Human Muscular Dystrophy: Elevation of Urinary Dimethylarginines. *Science* 1979; 203: 668

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin-Phenol reagents. *J Biol Chem* 1951; 193: 265-275

Ludmer PL, Selwyn AP, Shook TL, Wayne RR, Mudge GH, Alexander RW, Ganz P. Paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries. *N Engl J Med* 1986; 315(17): 1046-51

MacAllister RJ, Fickling SA, Whitley GS, Vallance P. Metabolism of methylarginines by human vasculature; implications for the regulation of nitric oxide synthesis. *Br J Pharmacol.* 1994;112(1):43-8

MacAllister RJ, Parry H, Kimoto M, Ogawa T, Russell RJ, Hodson H, Whitley GS, Vallance P. Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase. *Br J Pharmacol.* 1996; 119(8): 1533-40

MacAllister RJ, Rambausek MH, Vallance P, Williams D, Hoffmann KH, Ritz E.Concentration of dimethyl-L-arginine in the plasma of patients with end-stage renal failure. *Nephrol Dial Transplant* 1996b; 11(12): 2449-52

Masuda H, Goto M, Tamaoki S, Azuma H. Accelerated intimal hyperplasia and increased endogenous inhibitors for NO synthesis in rabbits with alloxan-induced hyperglycaemia. *Br J Pharmacol* 1999; 126(1): 211-8

Masuda H, Tsujii T, Okuno T, Kihara K, Goto M, Azuma H. Involvement of accumulated endogenous NOS inhibitors and decreased NOS activity in the impaired neurogenic relaxation of the rabbit proximal urethra with ischaemia. *Br J Pharmacol* 2001; 133(1): 97-106

Masuda H, Yano M, Sakai Y, Kihara K, Goto M, Azuma H.Roles of Accumulated Endogenous Nitric Oxide Synthase Inhibitors and Decreased Nitric Oxide Synthase Activity for Impaired Trigonal Relaxation With Ischemia. *J Urol* 2003; 170(4): 1415-1420

Matsunaga T, Weihrauch DW, Moniz MC, Tessmer J, Warltier DC, Chilian WM. Angiostatin inhibits coronary angiogenesis during impaired production of nitric oxide. *Circulation* 2002; 105(18): 2185-91

Matsuoka H, Itoh S, Kimoto, M, Kohno K, Tamai O, Wada Y, Yasukawa H, Iwami G, Okuda S, Imaizumi T. Asymmetrical dimethylarginine, an endogenous nitric oxide synthase inhibitor, in experimental hypertension. *Hypertension* 1997; 29: 242-247

McCabe TJ, Fulton D, Roman LJ, Sessa WC. Enhanced electron flux and reduced calmodulin dissociation may explain "calcium-independent" eNOS activation by phosphorylation. *J Biol Chem* 2000; 275(9): 6123-8

McDermott JR. Studies on the catabolism of NG-methylarginine, NG, N'G-dimethylarginine and NG, NG-dimethylarginine in the rabbit. *Biochem J* 1976; 154(1): 179-84

McDonald KK, Zharikov S, Block ER, Kilberg MS. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". *J Biol Chem* 1997; 272(50): 31213-6

Miano JM, Kelly LA, Artacho CA, Nuckolls TA, Piantedosi R, Blaner WS. all-Trans-retinoic acid reduces neointimal formation and promotes favorable geometric remodeling of the rat carotid artery after balloon withdrawal injury. *Circulation* 1998; 98(12): 1219-27

Miyake M, Kakimoto Y. Synthesis and degradation of methylated proteins of mouse organs: correlation with protein synthesis and degradation. *Metabolism* 1976; 25(8): 885-96

Miyazaki H, Matsuoka H, Cooke JP, Usui M, Ueda S, Okuda S, Imaizumi T. Endogenous nitric oxide synthase inhibitor: a novel marker of atherosclerosis. *Circulation.* 1999; 99(9): 1141-6

Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 1993; 329: 2002-2012

Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL, Isner JM. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 1998; 101(11): 2567-78

Murray-Rust J, Leiper J, McAlister M, Phelan J, Tilley S, Santa Maria J, Vallance P, McDonald N. Structural insights into the hydrolysis of cellular nitric oxide synthase inhibitors by dimethylarginine dimethylaminohydrolase. *Nat Struct Biol* 2001; 8(8): 679-83

Nakagomi S, Kiryu-Seo S, Kimoto M, Emson PC, Kiyama H. Dimethylarginine dimethylaminohydrolase (DDAH) as a nerve-injury-associated molecule: mRNA localization in the rat brain and its coincident up-regulation with neuronal NO synthase (nNOS) in axotomized motoneurons. *Eur J Neurosci* 1999; 11(6): 2160-6

Napoli C, Ignarro LJ. Nitric oxide and atherosclerosis. *Nitric Oxide* 2001; 5(2): 88-97

Naruse K, Shimizu K, Muramatsu M, Toki Y, Miyazaki Y, Okumura K, Hashimoto H, Ito T. Long-term inhibition of NO synthesis promotes atherosclerosis in the hypercholesterolemic rabbit thoracic aorta. PGH2 does not contribute to impaired endothelium-dependent relaxation. *Arterioscler Thromb* 1994; 14(5): 746-52

Neuville P, Yan Z, Gidlof A, Pepper MS, Hansson GK, Gabbiani G, Sirsjo A. Retinoic acid regulates arterial smooth muscle cell proliferation and phenotypic features *in vivo* and *in vitro* through an RARalpha-dependent signaling pathway. *Arterioscler Thromb Vasc Biol* 1999;19(6):1430-6

Norford D, Koo JS, Gray T, Alder K, Nettesheim P. Expression of nitric oxide synthase isoforms in normal human tracheobronchial epithelial cells *in vitro*: dependence on retinoic acid and the state of differentiation. *Exp Lung Res.* 1998;24(3):355-66

Ogawa T, Kimoto M, Sasaoka K Occurrence of a new enzyme catalyzing the direct conversion of NG,NG-dimethyl-L-arginine to L-citrulline in rats. *Biochemical and Biophysical Research Communications* 1987; 148(2): 671-7

Ogawa T, Kimoto M, Sasaoka K. Purification and properties of a new enzyme, NG,NG-dimethylarginine dimethylaminohydrolase, from rat kidney. *J Biol Chem* 1989; 264(17): 10205-9

Ohashi Y, Kawashima S, Hirata Ki, Yamashita T, Ishida T, Inoue N, Sakoda T, Kurihara H, Yazaki Y, Yokoyama M. Hypotension and reduced nitric oxide-elicited vasorelaxation in transgenic mice overexpressing endothelial nitric oxide synthase. *J Clin Invest* 1998; 102(12): 2061-71

Ohnishi M, Wada A, Tsutamoto T, Fujii M, Matsumoto T, Yamamoto T, Takayama T, Wang X, Kinoshita M. Endothelin stimulates an endogenous nitric oxide synthase inhibitor, asymmetric dimethylarginine, in experimental heart failure. *Clin Sci (Lond)* 2002; 103 Suppl 1: 241S-4S

Olken NM, Marletta MA. NG-methyl-L-arginine functions as an alternate substrate and mechanism-based inhibitor of nitric oxide synthase. *Biochemistry* 1993; 32(37): 9677-85

Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature* 1988; 333(6174): 664-6

Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987; 327(6122): 524-6

Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 1997; 100: 3131–3139

Petros A, Bennett D, Vallance P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. *Lancet* 1991; 338(8782-8783): 1557-8

Peunova N, Enikolopov G. Nitric oxide triggers a switch to growth arrest during differentiation of neuronal cells. *Nature* 1995; 375(6526): 68-73

Pipili-Synetos E, Sakkoula E, Maragoudakis ME. Nitric oxide is involved in the regulation of angiogenesis. *Br J Pharmacol* 1993; 108(4): 855-7

Preckel B, Kojda G, Schlack W, Ebel D, Kottenberg K, Noack E, Thamer V. Inotropic effects of glyceryl trinitrate and spontaneous NO donors in the dog heart. *Circulation* 1997; 96(8): 2675-82

Prescott LM, Jones ME. Modified methods for the determination of carbamyl aspartate. *Anal Biochem* 1969; 32(3): 408-19

Pritchard KA Jr, Groszek L, Smalley DM, Sessa WC, Wu M, Villalon P, Wolin MS, Stemerman MB. Native low-density lipoprotein increases endothelial cell nitric oxide synthase generation of superoxide anion. *Circ Res* 1995; 77: 510-8

Rakhit RD, Edwards RJ, Marber MS. Nitric oxide, nitrates and ischaemic preconditioning. *Cardiovasc Res* 1999; 43(3): 621-7

Rees DD, Palmer RMJ, Moncada S. Role of Endothelium-derived Nitric Oxide in the Regulation of Blood Pressure. *Proc Natl Acad Sci USA* 1989; 86: 3375-3378

Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest* 1998; 101(4): 731-6

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988; 239(4839): 487-91

Sander M, Chavoshan B, Harris SA, Iannaccone ST, Stull JT, Thomas GD, Victor RG. Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy. *Proc Natl Acad Sci USA* 2000; 97(25): 13818-23

Santa Maria J, Vallance P, Charles IG, Leiper JM. Identification of microbial dimethylarginine dimethylaminohydrolase enzymes. *Mol Microbiol* 1999; 33(6): 1278-9

Sarkar D, Vallance P, Harding SE. Nitric oxide: not just a negative inotrope. *Eur J Heart Fail* 2001; 3(5): 527-34

Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* 2000; 101(16): 1899-906

Schwartz S, Clare R, Devereux K, Sheung CF. Pharmacokinetics, disposition and metabolism of 546C88 (L-NG-methylarginine hydrochloride) in rat and dog. *Xenobiotica* 1997; 27(12): 1259-71

Shaish A, Daugherty A, O'Sullivan F, Schonfeld G, Heinecke JW. Beta-carotene inhibits atherosclerosis in hypercholesterolemic rabbits. *J Clin Invest* 1995; 96(4):2075-82

Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, Smithies O. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci U S A* 1996; 93(23): 13176-81

Silvestre JS, Mallat Z, Tamarat R, Duriez M, Tedgui A, Levy BI. Regulation of matrix metalloproteinase activity in ischemic tissue by interleukin-10: role in ischemia-induced angiogenesis. *Circ Res* 2001; 89(3): 259-64

Smith CL, Birdsey GM, Anthony S, Arrigoni FI, Leiper JM, Vallance P. Dimethylarginine dimethylaminohydrolase activity modulates ADMA levels, VEGF expression, and cell phenotype. *Biochem Biophys Res Commun* 2003; 308(4): 984-9.

Smith MA, Vasak M, Knipp M, Castellani RJ, Perry G. Dimethylargininase, a nitric oxide regulatory protein, in Alzheimer disease. *Free Radic Biol Med* 1998; 25(8): 898-902

Sorensen KE, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge DJ, Deanfield JE. Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. *J Clin Invest* 1994; 93(1): 50-5

Spanjaard RA, Lee PJ, Sarkar S, Goedegebuure PS, Eberlein TJ. Clone 10d/BM28 (CDCL1), an early S-phase protein, is an important growth regulator of melanoma. *Cancer Res* 1997; 57(22): 5122-8

Stamler JS, Lamas S, Fang FC. Nitrosylation. The prototypic redox-based signaling mechanism. *Cell* 2001; 106(6): 675-83

Stamler JS, Loh E, Roddy MA, Currie KE, Creager MA. Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. *Circulation* 1994; 89(5): 2035-40

Stuehr DJ, Cho HJ, Kwon NS, Weise MF, Nathan CF. Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc Natl Acad Sci U S A* 1991; 88(17): 7773-7

Stuhlinger MC, Tsao PS, Her J-H, Kimoto M, Balint RF and Cooke JP: Homocysteine Impairs the NO Synthase Pathway – Role of ADMA. *Circulation* 2001; 104(21): 2569-75

Sumeray MS, Rees DD, Yellon DM. Infarct size and nitric oxide synthase in murine myocardium. *J Mol Cell Cardiol* 2000; 32(1): 35-42

Tatchum-Talom R, Schulz R, McNeill JR, Khadour FH. Upregulation of neuronal nitric oxide synthase in skeletal muscle by swim training. *Am J Physiol Heart Circ Physiol* 2000; 279(4): H1757-66

Tokuo H, Yunoue S, Feng L, Kimoto M, Tsuji H, Ono T, Saya H, Araki N. Phosphorylation of neurofibromin by cAMP-dependent protein kinase is regulated via a cellular association of N(G),N(G)-dimethylarginine dimethylaminohydrolase. *FEBS Lett* 2001; 494(1-2): 48-53

Topper JN, Cai J, Falb D, Gimbrone MA Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci U S A* 1996; 93(19): 10417-22

Tousoulis D, Davies G, Tentolouris C, Crake T, Toutouzas P. Coronary stenosis dilatation induced by L-arginine. *Lancet* 1997; 349(9068): 1812-3

Tran CTL, Fox MF, Vallance P, Leiper JM. Chromosomal localisation, gene structure and expression pattern of *DDAH1*: Comparison with *DDAH2* and implications for evoloutionary origins. *Genomics* 2000; 68: 101-5

Tsao P, McEvoy LM, Drexler H, Butcher EC, Cooke JP: Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine. *Circulation* 1994; 89: 2176-82

Tsao PS, Buitrago R, Chan JR, Cooke JP. Fluid flow inhibits endothelial adhesiveness: Nitric oxide and transcriptional regulation of VCAM-1. *Circulation* 1996; 94: 1682-89

Tsikas D, Boger RH, Sandmann J, Bode-Boger SM, Frolich JC. Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox. *FEBS Lett* 2000; 478(1-2): 1-3

Uematsu M, Ohara Y, Navas JP, Nishida K, Murphy TJ, Alexander RW, Nerem RM, Harrison DG. Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *Am J Physiol* 1995; 269(6 Pt 1): C1371-8

Usui M, Matsuoka H, Miyazaki H, Ueda S, Okuda S, Imaizumi T. Increased endogenous nitric oxide synthase inhibitor in patients with congestive heart failure. *Life Sci* 1998; 62: 2425-30

Valkonen VP, Paiva H, Salonen JT, Lakka TA, Lehtimaki T, Laakso J, Laaksonen R. Risk of acute coronary events and serum concentration of asymmetrical dimethylarginine. *Lancet* 2001; 358(9299): 2127-8

Vallance P, Collier J, Moncada S. Effects of Endothelium-derived Nitric Oxide on Peripheral Arteriolar Tone in Man. *Lancet* 1989; 2: 997-1000

Vallance P, Leone A, Calver A, Collier J, Moncada S. Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* 1992; 339: 572-5

Varani J, Jones J, Dame M, Sulavik C, Gibbs DF, Johnson KJ. Effects of all-trans retinoic acid on neutrophil-mediated endothelial cell injury *in vitro* and immune complex injury in rats. *Am J Pathol.* 1991; 139(4): 901-9

Vita JA, Treasure CB, Nabel EG, McLenachan JM, Fish RD, Yeung AC, Vekshtein VI, Selwyn AP, Ganz P. Coronary vasomotor response to acetylcholine relates to risk factors for coronary artery disease. *Circulation* 1990; 81(2): 491-7

von der Leyen HE, Gibbons GH, Morishita R, Lewis NP, Zhang L, Nakajima M, Kaneda Y, Cooke JP, Dzau VJ: Gene therapy inhibiting neointimal vascular lesion: *In vivo* transfer of endothelial cell nitric oxide synthase gene. *Proc Natl Acad Sci USA* 1995; 92: 1137-41

Wahrendorf J, Hanck AB, Munoz N, Vuilleumier JP, Walker AM. Vitamin measurements in pooled blood samples. *Am J Epidemiol* 1986; 123(3): 544-50

Wang S, Wright G, Geng W, Wright GL. Retinol influences contractile function and exerts an anti-proliferative effect on vascular smooth muscle cells through an endothelium-dependent mechanism. *Pflugers Arch* 1997; 434(6): 669-77

Weidinger FF, McLenachan JM, Cybulsky M, Gordon JM, Rennke HA, Hollenberg NK, Ganz P, Cooke JP. Persistent dysfunction of regenerated endothelium following balloon angioplasty of rabbit iliac artery. *Circulation* 1990; 81: 1667-1679

Wever RM, Luscher TF, Cosentino F, Rabelink TJ. Atherosclerosis and the two faces of endothelial nitric oxide synthase. *Circulation* 1998; 97: 108-12

Wiegman PJ, Barry WL, McPherson JA, McNamara CA, Gimple LW, Sanders JM, Bishop GG, Powers ER, Ragosta M, Owens GK, Sarembock IJ. all-Trans-retinoic acid limits restenosis after balloon angioplasty in the focally atherosclerotic rabbit: a favorable effect on vessel remodeling. *Arterioscler Thromb Vasc Biol* 2000; 20: 89-95

Williams RL, Risau W, Zerwes HG, Drexler H, Aguzzi A, Wagner EF. Endothelioma cells expressing the polyoma middle T oncogene induce hemangiomas by host cell recruitment. *Cell* 1989; 57(6): 1053-63

Wojcik C, Wilk S. Changes in proteasome expression and activity during differentiation of neuronal precursor NTera 2 clone D1 cells. *Neurochem Int* 1999; 34(2): 131-6

Yoo JH, Lee SC. Elevated levels of plasma homocyst(e)ine and asymmetric dimethylarginine in elderly patients with stroke. *Atherosclerosis* 2001; 158(2): 425-30

Zeiher AM, Drexler H, Saurbier B, Just H. Endothelium-mediated coronary blood flow modulation in humans. Effects of age, atherosclerosis, hypercholesterolemia, and hypertension. *J Clin Invest* 1993; 92(2): 652-62

Zeiher AM, Krause T, Schachinger V, Minners J, Moser E. Impaired endothelium-dependent vasodilation of coronary resistance vessels is associated with exercise-induced myocardial ischemia. *Circulation* 1995; 91(9): 2345-52

Zeisel SH, DaCosta KA, Fox JG. Endogenous formation of dimethylamine. *Biochem J* 1985; 232: 403-8

Zhang AQ, Mitchell SC, Barrett T, Ayesh R, Smith RL. Fate of dimethylamine in man. *Xenobiotica* 1994; 24: 379-87

Zhang AQ, Mitchell SC, Smith RL. Dimethylamine in human urine. *Clin Chim Acta* 1995; 233: 81-8

Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bicknell R. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* 1997; 99(11): 2625-34

Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, Geppetti P, Ledda F. Nitric oxide mediates angiogenesis *in vivo* and endothelial cell growth and migration *in vitro* promoted by substance P. *J Clin Invest* 1994; 94(5): 2036-44

Zoccali C, Bode-Boger S, Mallamaci F, Benedetto F, Tripepi G, Malatino L, Cataliotti A, Bellanuova I, Fermo I, Frolich J, Boger R. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. *Lancet* 2001; 358(9299): 2113-7

Zoccali C, Mallamaci F, Maas R, Benedetto FA, Tripepi G, Malatino LS, Cataliotti A, Bellanuova I, Boger R; CREED Investigators. Left ventricular hypertrophy, cardiac remodeling and asymmetric dimethylarginine (ADMA) in hemodialysis patients. *Kidney Int* 2002; 62(1): 339-45

Supplements

Abbreviations

ACh	acetylcholine
ADMA	asymmetric dimethylarginine
ApoE	apolipoprotein E
atRA	all-*trans*-retinoic acid
BH4	tetrahydrobiopterin
CO	cardiac output
Cr	creatinine
CVP	central venous pressure
DDAH	dimethylarginine dimethylaminohydrolase
EDRF	endothelium derived relaxing factor
eNOS	endothelial NOS
FAD	flavin adenine dinucleotide
FMN	flavin adenine mononucleotide
HPLC	high performance liquid chromatography
iNOS	inducible NOS
L-NAME	NG-nitro-L-arginine methyl ester
L-NMMA	NG-monomethyl-L-arginine
LV	left ventricle (ventricular)
MAP	mean arterial pressure
NADPH	nicotinamide adenine dinucleotide phosphate
nNOS	neuronal NOS
NO	nitric oxide
NOS	nitric oxide synthase

PAEC	primary arterial endothelial cell
PCR	polymerase chain reaction
PRMT	protein arginine methyltransferase
RT	reverse transcription
SDMA	symmetric dimethylarginine
SGC	soluble guanylate cyclase
SGMP	cyclic guanosine monophosphate
SVR	systemic vascular resistance
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell